Mapping QTL for salinity tolerance and its related physiological traits in barley (*Hordeum vulgare* L.)

By

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Declarations

The thesis contains no material which has been accepted for a degree or diploma by a University or any other institutions, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Publications

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Statement of co-authorship

This thesis was completed during the course of my enrolment in a PhD degree in the School of Land and Food at the University of Tasmania. This thesis contains no experimental results that have previously presented for any degree at this or other Institution.

This thesis contains one literature review chapter and five research chapters. One section in the literature review chapter (Chapter 2) has been published as a book chapter. Results described in the five research chapters have been or will be published in different journals.

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We agree with the above stated "contribution of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Student Signature:

Primary Supervisor Signature:

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List of Abbreviations

$^1O_2$ ----- singlet oxygen

ABA ----- abscisic acid

ABI2 ----- ABA insensitive 2

AL ----- aperture length

ANP1 ----- Arabidopsis NPK1-related protein kinase1

AO ----- antioxidant

AOX ----- alternative oxidase

AP2/ERF ----- apetala2/ ethylene response factor

APX ----- ascorbate peroxidase

AsA ----- ascorbic acid

AW ----- aperture width

BAC ----- bacterial artificial chromosome

bHLH ----- basic helix–loop–helix

BR ----- brassinosteroid

bZIP ----- basic leucine zipper

CaM ----- calmodulin

CAM ----- Crassulacean Acid Metabolism

Cas ----- CRISPR-associated

CAT ----- catalase

CBLs ----- calcineurin B-like proteins

CDPKs ----- calcium-dependent protein kinases

CIPKs ----- CBL-interacting protein kinases

cM ----- centi-Morgans

CRISPR ----- clustered regularly interspaced short palindromic repeats

DArT ----- Diversity Array Technology

DBS ----- double strand breaks

DH ----- double haploid

DHA ----- dehydroascorbate

DHAR ----- dehydroascorbate reductase

DREB ----- dehydration responsive element binding protein

DT ----- drought tolerance

EC ----- electrical conductivity

EE ----- ear emergency

ERF ----- ethylene response factor

ETC ----- electron transport chain

F_2 ----- second generation

GA ----- gibberellic acid

Gb ----- gigabases

GBS ----- genotype by sequencing

GCL ----- guard cell length

GCV ----- guard cell volume

GCW ----- guard cell width

GEBVs ----- genomic estimated breeding values

GLM ----- general linkage model

GM ----- genetic modified

GPX ----- glutathione peroxidase

GR ----- glutathione reductase

gRNA ----- guide RNA

GS ----- genome selection

GSH ----- glutathione

GSSG ----- glutathione

GWAS ----- genome wide association study

H^+-PPase ----- H^+-pyrophosphatase

H_2O_2 ----- hydrogen peroxide

HDR ----- homology-directed repair

HKT1 ----- high-affinity K+ transporter1

HO/OH·-hydroxyl radical

HSFs ----- heat shock factors

IM ----- interval mapping

Indel ----- insertion and deletion

JA ----- jasmonic acid

KORC ----- K^+ outward rectifier channels

LD ----- linkage disequilibrium

LEA ----- late embryogenesis abundant

LOD ----- logarithm of the odds

MAF ----- minor allele frequency

MAGIC ----- Multi-parent Advanced Generation Inter-Cross

MAPK ----- mitogen-activated protein kinase

MAS ----- marker assisted selection

MCMC ----- Markov Chain Monte Carlo

MDA ----- monodehydroascorbate

MDAR ----- monodehydroascorbate reductase

MJ ----- Methyl jasmonate

MLM ----- mixed linkage model

MQM ----- multiple QTL model

mtETC ----- mitochondria electron transport chain

NBT ----- nitroblue tetrazolium

NDPK ----- nucleoside diphosphate kinase

NGS ----- next generation sequencing

NHEJ ----- non-homologous end-joining

NHX ----- Na^+/H^+ exchanger

NILs ----- near isogenic lines

NO ----- nitric oxide

NPR1 ----- pathogenesis-related genes1

NSCCs ----- nonselective cation channels

O_2 ----- oxygen

$O_2^{\bullet -}$ ----- superoxide anion

OST1 ----- open stomata 1

P5CR ----- pyrroline-5-carboxylate reductase

P5CS ----- pyrroline-5-carboxylate synthetase

PAR ----- photosynthetically active radiation

PAs ----- phosphatidic acids

PBS ----- phosphate buffer solution

PC ----- proline content

PCA ----- Principle component analysis

PCD ----- programmed cell death

PDK1 ----- phosphoinositide-dependent kinase1

PI3P ----- Phosphatidylinositol 3-phosphate

PLC/D-PA ----- phospholipase C/D – phosphatidic acid

PLDα1 ----- phospholipase Dα1

POD ----- peroxidase

POPSEQ ----- population sequencing

PPI ----- protein phosphatase interaction

PQ ----- plastoquinone

PSI/II ----- photosystem I/II

QA ----- quinone A

Q-Q ----- quantile-quantile

QTL ----- quantitative trait loci

RBOH ----- Respiratory Burst Oxidase Homologues

RCD1 ----- radical-induced cell death

RH ----- relative humidity

RILs ----- recombinant inbred lines

RMO ----- relative moisture

ROS ----- Reactive oxygen species

RWC ----- relative water content

SA ----- salicylic acid

SA (in Chapter 5) ----- stomatal pore area

SAR ----- system acquired resistance

SCL ----- subsidiary cell length

SCV ----- subsidiary cell volume

SCW ----- subsidiary cell width

SD ----- stomatal density

SI ----- stomatal index

SOD ----- superoxide dismutase

SOS ----- Salt overly sensitive

SSR ----- Simple Sequence Repeat

ST ----- salinity tolerance

TALEN ----- transcription activator like effector nucleases

TFs ----- transcriptional factors

TGA ----- TGACG-sequence-specific binding-protein

tylAPX ----- thylakoid ascorbate peroxidase

UQ ----- ubiquinone

Vitamin E ----- a-Tocopherol

V-ppase ----- vacuolar inorganic H^+-pyrophosphatase

WGS ----- whole genome shotgun

WSC ----- water-soluble carbohydrate concentration

XOD ----- xanthine oxidase

ZFN ----- zinc finger nucleases

Abstract

Soil salinity is one of the major abiotic stresses which severely affect crop yield and restrict the utilization of agricultural land. Breeding salt tolerant crops has become one of the top priorities, as salinity is causing global food issues due to the large arable and saline area which are not suitable for cropping. Salinity stress is considered to be composed of two phases at the whole-plant level: a rapid osmotic stress which reduces shoot growth, and slower ionic stress which accelerates senescence of older leaves due to elevated leaf Na^+ content. Osmotic stress affects plant growth by reducing cell expansion and elongation rates, which leads to smaller and thicker leaves and down-regulated photosynthesis by reducing stomatal aperture. Plants employ numerous mechanisms to adapt to saline conditions such as Na^+ exclusion from uptake, control of xylem Na^+ loading and/or its retrieval from the shoot, efficient vacuolar Na^+ sequestration, efficient osmotic adjustment, and ROS detoxification. Since many traits underlying adaption to stress are quantitative and controlled by multiple genetic pathways, a wide variety of genes are implicated in salinity tolerance.

Molecular marker assisted selection (MAS) has been successfully used in barley breeding programs, particularly for traits which are easily affected by environments. However, less progress has been made in salt tolerance due to the lack of efficient QTL that can be used MAS. The objective of this study were (i) to detect QTL controlling salinity tolerance and some physiological traits using different barley populations; (ii) to investigate the contribution of different physiological traits to plant overall salinity tolerance; (iii) to study the relationships between QTL for agronomic and physiological traits and those for plant drought and salinity tolerance using QTL mapping; (iv) to fine map a QTL for salinity tolerance which has been identified in our previous work.

ROS detoxification is one of the salinity tolerance mechanisms in plants, which includes enzymatic and non- enzymatic scavenging. To investigate the role of major antioxidant (AO) enzymes in plant salinity tolerance and whether it is suitable for using as selection criteria of salinity tolerance, two barley varieties with contrast salinity tolerance (TX9425 & Naso Nijo) were firstly used to evaluate the activity of major AO enzymes in different leaves and at different times after salt treatment. Our results showed that AO enzyme activities had strong tissue- and time-specificity. A further study was conducted using six barley varieties contrasting in salinity tolerance (TX9425, YYXT, CM72, Naso Nijo, Franklin and Gairdner).

AO enzyme activities and proline contents were measured in the third leaves of seedlings after plants were treated with 240 mM NaCl for 10 days. No significant correlation was revealed between leaf AO activity and either plant grain yield or plant survival rate under salt stress. Although salinity induces changes in leaf AO enzyme activities, the change cannot be used as biochemical indicator in breeding for salinity tolerance.

A double haploid (DH) population from the cross of TX9425 (a Chinese landrace variety with both salinity and drought tolerance) and Franklin (sensitive to both salinity and drought) was used to identify QTL for salinity and drought tolerance. One QTL for salinity tolerance on 7H based on plant survival under salt stress and two QTL for drought tolerance on 2H and 5H using leaf wilting under drought stress conditions were identified from this population. The QTL for proline accumulation under both salinity and drought stresses were located on different positions to those for drought and salinity tolerance, indicating no relationship with plant tolerance to either of these stresses. It was also shown that proline accumulation under stresses was merely a symptom of plant damage thus not to be a useful selection criterion for either drought or salinity tolerance.

Stomata regulate photosynthesis and transpiration, which are critical for plant responses to abiotic stresses such as salinity. To understand the genetic basis controlling salinity tolerance and stomatal parameters, a DH population from the cross of CM72 and Gairdner was used to detect QTL underlying these traits. Total of 11 significant QTL (LOD > 3.0) and 11 tendency QTL (2.5 < LOD < 3.0) were investigated distributing on all different chromosomes except for 5H. Co-localization of QTL for biomass with that for intercellular CO_2 concentration, transpiration rate and stomatal conductance was found under control condition. A QTL for biomass also co-located with one for transpiration rate under salinity stress. The QTL for salinity tolerance also co-localised with QTL for grain yield on chromosome 3H. The lack of major QTL for gas exchange and stomatal traits under control and saline conditions indicates a complex relationship between salinity and leaf gas exchange and the fact that these complex quantitative traits are under the control of multiple genes.

A wide range of barley accessions were used to detect genetic variations through genome wide association study (GWAS). The 206 barley accessions collected worldwide were genotyped with 408 Diversity Arrays Technology (DArT) markers and evaluated for salinity stress tolerance using plant damage scores under salinity stress – a reliable method developed

in our previous work. GWAS for salinity tolerance had been conducted through a general linkage model (GLM) and a mixed linkage model (MLM) based on population structure and kinship. A total of 24 significant marker-trait associations were identified. A QTL on 4H with the nearest marker of bpb-9668 was consistently detected in all different methods. This QTL has not been reported before and is worth to be further confirmed with bi-parental population.

A major QTL for salinity tolerance was identified in the DH population from the cross between TX9425 and Naso Nijo in our previous study. This QTL explained more than 45% of the phenotypic variation. Further fine mapping has been conducted to this population. A new marker was identified to be more closely linked to this gene, determining more than 70% of the phenotypic variation. Near isogenic lines have been developed for further fine mapping, physiological studies and the identification of gene(s).

In conclusion, several QTL were identified for salinity tolerance and its related physiological traits, including Na^+ content, proline content, stomata pore area, leaf temperature and transpiration rate. The QTL for salinity tolerance on 3H from the cross of CM72 and Gairdner was located at the same position as that for grain yield under salinity stress. Most of the QTL for physiological traits were located at different positions to those for salinity tolerance. One new QTL for salinity tolerance was detected through genome wide association studies and this QTL will be further confirmed by bi-parental populations. We have also fine mapped a major QTL that was reported earlier to less than 2 cM. Near isogenic lines were constructed for further fine mapping and studies on gene expression. ROS antioxidants were found to be affected by numerous factors such as leaf age, salt concentration and treatment time, thus can’t be used as indirect selection criteria for salinity tolerance.

Chapter 1 General introduction

1.1 Plant response to salinity stress and its tolerance mechanisms

Soil salinization is a growing problem for agriculture production worldwide (Deinlein et al. 2014). Based on the information from FAO (The United Nations Food and Agriculture Organization), more than 400 million hectares of the irrigated land are affected by soil salinity, which mainly results from global climate changes and irrigation practices (Koohafkan 2012; Rengasamy 2010; Roy et al. 2014). Salinity stress disrupts plant metabolisms and decreases photosynthetic efficiency, leading to slower growth rates, reduced tillering and decreased crop yield ultimately (Munns and Tester 2008; Roy et al. 2014). At the whole-plant level, salinity stress is considered to be composed of two phases: a rapid osmotic stress which affects the growth of new leaves, and slower ionic stress which accelerates senescence of older leaves due to elevated leaf Na^+ content (Munns and Tester 2008). This can be distinguished by measuring effects within minutes to a few days after salinity treatment (Roy et al. 2014). Within minutes of salt treatment, rapid responses include stomatal closure, increased leaf temperature and reduced shoot elongation (Rajendran et al. 2009; Sirault et al. 2009). After few days to weeks, premature senescence of older leaves can be observed where salt accumulation reaches to a toxic concentration (Munns and Tester 2008; Roy et al. 2014). Plants employ mechanisms to mitigate osmotic stress by osmotic adjustment and reducing water loss, while plants decrease ionic stress mainly by Na^+ exclusion from uptake or tissue tolerance through efficient vacuolar Na^+ sequestration (Deinlein et al. 2014; Munns and Tester 2008).

Numerous mechanisms/systems are involved in plant salinity tolerance such as antioxidants and detoxifying system, network of ion transport, accumulation of compatible solutes, transcription factors and hormones regulation (Bahmani et al. 2015). To date, plant sensory mechanism of salt stress including both hyperosmotic and Na^+ sensors remains elusive (Deinlein et al. 2014). A rapid rise of cytosolic Ca^{2+} in roots was observed within seconds after salt treatment or mannitol, which indicated that hyperosmotic stress maybe sensed by Ca^{2+} channel (Knight et al. 1997; Tracy et al. 2008). Other second messengers such as ROS, annexins were reported to be linked to NaCl-induced Ca^{2+} signalling (Jiang et al. 2013b; Laohavisit et al. 2013). Ca^{2+} kinases such as calcium-dependent protein kinases (CDPKs), calcineurin B-like proteins (CBLs), CBL-interacting protein kinases (CIPKs) may transduce

the hyperosmotic signal to down-stream gene transcription (Boudsocq and Sheen 2013; Harmon et al. 2000; Weinl and Kudla 2009). Many transcription factors were suggested to link salt sensory pathways to tolerance responses, and participate in transcriptional regulation of salinity tolerance such as apetala2/ ethylene response factor (AP2/ERF), basic helix–loop–helix (bHLH), basic leucine zipper (bZIP), WRKY, MYB, and NAC families (Cui et al. 2013; Deinlein et al. 2014; Jiang and Deyholos 2009; Jiang et al. 2009; Kasuga et al. 1999; Tran et al. 2004; Yang et al. 2009a). Most transcriptional changes occur about 3 hours after exposure to high salinity stress in Arabidopsis (Geng et al. 2013). Transcriptional regulation of stress response genes in plants may be affected by plant hormones in some extent, including abscisic acid (ABA), gibberellic acid (GA), jasmonate (JA), brassinosteroid (BR) and ethylene (Dinneny et al. 2008; Geng et al. 2013; Jiang et al. 2013a; Kilian et al. 2007). Ethylene was recently shown to promote soil-salinity tolerance via improved Na^+/K^+ homeostasis mediated by respiratory burst oxidase homolog F (RBOHF)-dependent regulation of Na accumulation and RBOHF-independent regulation of K accumulation (Jiang et al. 2013a).

A network of Na^+ and K^+ transport processes has been reported to be one of the key mechanisms for salinity tolerance in many studies. Several crucial determinants of cellular Na^+/K^+ homeostasis such as high-affinity K^+ transporter1 (HKT1), salt overly sensitive1 (SOS1) and Na^+/H^+ exchanger (NHX) have been identified, where *HKT1* and *SOS1* are responsible for ion flux across the plasma membrane while *NHX* controlling flux across the tonoplast membrane into the vacuole (Deinlein et al. 2014; Hasegawa 2013; Mickelbart et al. 2015; Munns and Tester 2008). Na^+ enter root epidermal and cortical cells through nonselective cation channels (NSCCs) which induces membrane depolarization (Demidchik and Maathuis 2007). Membrane depolarization further activates K^+ outward rectifier channels (KOR) (Shabala and Cuin 2008). High cytosolic Ca^{2+} activates CBL9. CBL9 interacts with CIPK26 which targets RbohF, leading to the production of ROS (Drerup et al. 2013). Increased cytosolic Ca^{2+} and ROS induce the accumulation of ABA (Ismail et al. 2014). These early signalling pathways activate the expression of genes/mechanisms involved in cellular detoxification, including HKT, NHX, SOS transport mechanisms as well as osmotic adjustment strategies (Deinlein et al. 2014). Na^+ distribution is also regulated in a tissue-specific manner by controlling xylem Na^+ loading and/or its retrieval from the shoot (Deinlein et al. 2014). Apart from excluding Na^+ from plant to saline environment, some plants (like succulent halophytes) have swollen internal vacuoles to sequester salt into

internal compartment, or possess epidermal bladder cells (salt bladders) to deposit salt (Shabala et al. 2014). In addition, the accumulation of some organic osmolytes, such as proline and glycine betaine, play important roles in maintaining the low intracellular osmotic potential in plants and mitigating the harmful effects of salinity stress (Deinlein et al. 2014; Verslues et al. 2006).

Salinity tolerance is a quantitative trait controlled by multiple genetic pathways and a wide range of genes are implicated (DeRose-Wilson and Gaut 2011). Salinity stress induced changes in plants have been used to screen plants for tolerance, discover novel genes contributing to salt stress tolerance and breed salinity tolerant crops through modern molecular breeding technologies (Julkowska and Testerink 2015).

1.2 Genetic approaches for breeding salinity tolerant crops

The ultimate aim of salinity tolerance research is to increase plant yield under stress conditions. A wide range of biotechnologies are available for gene discovery and salinity resistant crop breeding (Roy et al. 2014). Classical breeding refers to the use of interbreeding of close relatives to produce new cultivars with desirable traits. However, it takes long time to select and evaluate useful individuals (He et al. 2014; Tester and Langridge 2010). Modern breeding programs become more effective with the help of molecular biotechnologies, including marker assisted selection (MAS) and genetic transformation. A great number of genes have been overexpressed improving salinity tolerance performance in crops (reviewed by Roy et al. (2014). When it comes to breeding and the application of genetic modified (GM) crops, however, it is hindered due to the controversy on food safety issues and environment impacts (He et al. 2014; Nicolia et al. 2014). MAS refers to the application of molecular markers for indirect selection on traits of interest in crop improvement, which is a more promising tool (He et al. 2014). In wheat, two genes for Na^+ exclusion (*Nax1*, *Nax2*) were introduced from tetraploid durum wheat into hexaploid bread wheat by interspecific crossing and marker assisted selection, where decreased leaf Na^+ concentration were observed in hexaploid plants containing one or both genes (James et al. 2011). *Nax2* (*TmHKT1;5-A*) was also introduced into a commercial durum wheat by MAS, which showed a 25% increase in yield when grown on saline soils compared to near-isogenic lines without *TmHKT1;5-A* (Munns et al. 2012).

The vital bases of successful breeding with MAS are the selection of appropriate markers and in-depth knowledge of genetic traits which depends on QTL mapping (Ashraf and Foolad 2013). QTL mapping refers to the statistical linkage analysis between genetic markers and traits of interest using populations such as doubled haploid (DH) populations, early generation segregating populations (F_2) and recombinant inbred lines (RILs) derived from parental varieties (Takeda and Matsuoka 2008). QTL mapping can help discover mechanisms of salinity tolerance from the aspect of genetics and provide effective molecular markers for MAS. The massive accumulation of QTL information leads to meta-analysis and QTL pyramiding. QTL meta-analysis statistically combines a huge amount of molecular and phenotypic data, which facilitates the comparison of QTL locations among different populations and prioritises candidate genes (Barabaschi et al. 2016; Wu and Hu 2012). Meanwhile, meta-data collections can eliminate information redundancy and highlight the missing data. Because plant salinity tolerance is a complicated trait and different tissues are adapted for different or specific functions, thereby plant breeders should pyramid a range of traits/genes and combine several mechanisms to improve salinity tolerance (Bahmani et al. 2015; Roy et al. 2014). Ultimate QTL pyramiding, relying on QTL mapping coupled with MAS, allows the introduction of multiple beneficial traits into an elite variety to breed salinity tolerant crops (Takeda and Matsuoka 2008).

The availability of next generation sequencing (NGS), bioinformatics resources and phenotyping platforms moves traditional plant breeding to a “next generation breeding” (Barabaschi et al. 2016). NGS leads to a high throughput for DNA sequencing and genotype by sequencing (GBS) for gene discovery and marker development. GBS is a novel application of NGS for genotyping and SNP discovering in crop genome and population (He et al. 2014). A recent strategy based on sequencing all genotypes of a segregating population (POPSEQ) was conducted for developing high density genetic maps (Mascher et al. 2013). Sequence based markers (SNP) from NGS can be used for genome wide association study (GWAS), MAS and genome selection (GS). GWAS exploits linkage disequilibrium (LD) between genetic markers and phenotype traits of interest across all chromosomes in natural populations, which benefits from more recombination events in large collections of diverse germplasms (Takeda and Matsuoka 2008). GWAS in many plant species have been reviewed recently (Huang and Han 2014; Ogura and Busch 2015). Several factors should be taken into account when employing GWAS such as LD levels and population structure. LD levels vary among different species (e.g. selfing and outcrossing), while population structure may cause

false associations (Barabaschi et al. 2016). Besides, the accuracy of phenotypic data, sample size and heritability of traits also affect the power of detecting significant associations (Korte and Farlow 2013). Association mapping is traits oriented, while genome selection mainly relies on DNA sequence polymorphism (Takeda and Matsuoka 2008). GS exploits all available markers as genome wide prediction of breeding value (Barabaschi et al. 2016). All loci, haplotypes and markers effects will be evaluated across the entire genome to calculate the genomic estimated breeding values (GEBVs) in a population for pre-breeding purpose (Heffner et al. 2009; Nakaya and Isobe 2012). The accuracy of GEBVs predictions are also influenced by LD levels and marker density of the genome (Barabaschi et al. 2016).

Another alternative technology based on recombination and genome editing has also been employed for plant improvement. Genome editing refers to targeted modification of genes (i.e. precision mutagenesis), which depends on accurate genome sequence information for precise determination of target site. Earlier genome editing methods were based on zinc finger nucleases (ZFN) and transcription activator like effector nucleases (TALEN) systems (Ainley et al. 2013; Palmgren et al. 2015). A newly developed system CRISPR/Cas9 was developed from the bacterial and archaeal clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system. This system is based on CRISPR-associated (Cas) nuclease and a single guide RNA (gRNA) from the type II bacterial CRISPR (Belhaj et al. 2013; Roy et al. 2014; Shan et al. 2014). Genome editing relies on the induction of double strand breaks (DBS) at specific genomic site, which triggers cellular repair pathways through non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathway (Barabaschi et al. 2016). In CRISPR/Cas9 system, careful selection of the gRNA sequence is very crucial, and the target sequence can be designed in a more predictable way compared to TALEN or ZFN due to its RNA-DNA interactions (Bortesi and Fischer 2015). Hundreds of papers have been reported for CRISPR/Cas9 system such as their application in wheat (Zhang et al. 2016) and rice (Zhang et al. 2014), which may be widely used in the future due to its efficiency. Availability of genome sequence of crops will facilitate genome editing approaches for plant improvement. Ultimately, both molecular breeding and advanced biotechnologies should help develop crops with enhanced performances.

1.3 Barley is the most salinity tolerant cereal

Barley is one of the most important cereal crops worldwide, and also the most salt tolerant cereal (Munns and Tester 2008). Cultivated barley originated from its wild progenitor *Hordeum vulgare ssp. Spontaneum*, and domesticated within the Fertile Crescent and Tibet (Badr et al. 2000; Dai et al. 2012; Kilian et al. 2006). Barley is indispensable to malting and brewing industries and also serves as a staple food in some area of the world due to its widely adaption to salinity, drought and high altitude (Baik and Ullrich 2008). It is more stress tolerant than its close relative wheat (Nevo et al. 2012). Both genetic diversity and adaption to broad conditions resulted in a rich gene pool of barley (Nevo and Chen 2010). Barley has always been considered a model for plant genetic research. It is a diploid crop with a large haploid genome of 5.1 gigabases (Gb), and is also an inbreeding and temperate crop (Mayer et al. 2012). Compared to *Arabidopsis*, barley has more advantages for salinity tolerance research such as broader genetic diversity, higher salinity tolerance and more direct contribution to agriculture.

In barley, traditional bi-parental QTL mapping has been widely used for the dissection of salinity tolerance and the identification of tolerance genes. Numerous QTL for salinity tolerance have been detected using a wide variety of agronomic and physiological traits as selection criteria for salt tolerance including plant survival (Fan et al. 2015; Xu et al. 2012; Zhou et al. 2012), Na^+ exclusion (Shavrukov et al. 2010), tissue ion content (Xue et al. 2009), water soluble carbohydrate and chlorophyll content (Siahsar and Narouei 2010), seed germination (Witzel et al. 2010), yield and agronomic traits (Ellis et al. 2002; Xue et al. 2009). Genome wide association mapping has also been used for detecting genetic variations controlling salinity tolerance (Long et al. 2013).

NGS provides an opportunity for generating a reference genome sequence at a relatively low cost. A physical, genetic and functional barley genome sequence assembly has been released mainly using bacterial artificial chromosome (BAC) based approaches and whole genome shotgun (WGS) strategy (Mayer et al. 2012). A physical map of 4.98 Gb was developed and more than 3.9 Gb has been anchored to a high resolution genetic map (Mayer et al. 2012). Reference sequenced genome with whole-genome shotgun assemblies is highly useful for gene discovery and genomics-assisted breeding, but it fails to link nearby contigs and provide a linear order of them (Mascher et al. 2013). A population sequencing (POPSEQ) strategy has

been employed to reconstruct the chromosomal organization of the gene space of barley genome (Mascher et al. 2013). Individuals from a segregating population were sequenced and a genetically anchored linear assembly of gene space was produced. Crop breeding can benefit from their sequenced genome in many ways such as the availability of high density molecular markers, which can be used for fast mapping desirable trait and the identification of candidate genes. Once traits/genes are characterized, they can be introgressed into elite variety through MAS (Bolger et al. 2014).

1.4 Aims of this study

Several key components of plant salinity tolerance network have been identified, however, there are still lots of gaps that need to be filled (Deinlein et al. 2014). Many questions need to be answered on the physiological and molecular mechanisms that control barley salt tolerance. What are the vital mechanisms for barley salinity tolerance? Why is screening barley for salt tolerance so difficult? Which traits of salinity tolerance can be combined to increase barley salinity tolerance? Therefore, the objectives of the work in this thesis are to:

(1) discover reliable, convenient and stable screening methods for barley salinity tolerance;
(2) investigate the relationships between salinity tolerance and physiological traits, and find out the possible mechanisms of barley salinity tolerance through genetic approaches (QTL mapping);
(3) identify candidate QTL/genes for salinity tolerance through QTL mapping and GWAS;
(4) fine map a QTL for salinity tolerance which has been identified in our previous work.

1.5 Outline of chapters

Chapter 2 -- Literature review about ROS production, scavenging and signalling under salinity stress

Chapter 3 -- Antioxidant activity in salt-stressed barley leaves: evaluating time- and age-dependence and suitability for the use as a biochemical marker in breeding programs

Chapter 4 -- Using QTL mapping to investigate the relationships between abiotic stress tolerance (drought and salinity) and agronomic and physiological traits

Chapter 5 -- QTL for stomatal and photosynthetic traits related to salinity tolerance in barley

Chapter 6 -- Genome-wide association study reveals a new QTL for salinity tolerance in barley (*Hordeum vulgare* L.)

Chapter 7 -- Fine mapping of a major QTL for salt tolerance on 2H in barley

Chapter 8 -- General conclusions and Recommendations

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Fan, Y., Bose, J., Zhou, M., Shabala, S. 2015. ROS production, scavenging and signalling under salinity stress. In: Wani, S. H., Hossain, M. A., eds. Managing salinity tolerance in plants: molecular and genomic perspectives, CRC Press, Taylor & Francis Group

Chapter 3 Antioxidant activity in salt-stressed barley leaves: evaluating time- and age-dependence and suitability for the use as a biochemical marker in breeding programs

3.1 Introduction

As one of the major abiotic stresses, soil salinity severely affects agricultural productivity. It is estimated that more than 20% of global irrigated land is affected by salinity (Yeo 1999) and around two million hectares of broadacre farmland is affected by dryland salinity, with a further six million hectares at risk. Thus, the need to breed salt tolerant crops is evident. Two main approaches are used for improving plant salt tolerance: (1) traditional breeding to explore natural genetic variations through direct selection under stress or through mapping quantitative trait loci (QTL) and a subsequent marker-assisted selection (MAS) (Flowers 2004; Lindsay et al. 2004) and (2) genetic manipulation techniques to produce transgenic plants with new genes or different expression levels of existing genes to improve plant salt tolerance (Cuin and Shabala 2007b). In recent decades plant genotyping has progressed rapidly while phenotyping remains a bottleneck for breeding due to a lack of understanding of salt tolerance mechanisms and reliable, rapid, inexpensive and convenient screening techniques (Chen et al. 2005; Munns and James 2003; Zhu 2000).

Soil salinity affects plants in two phases: rapid osmotic stress which reduces shoot growth and slower ionic stress which hastens senescence of older leaves due to elevated leaf Na^+ content (Munns and Tester 2008). Osmotic stress impacts plant growth by reducing cell expansion and elongation rates, which leads to smaller and thicker leaves, and down-regulating photosynthesis by immediately reducing stomatal aperture (Bradford 1976). The main site of Na^+ toxicity in most plants is the leaf blade (Munns 2002). Plants response to ionic stress through Na^+ exclusion, which requires a good control of net delivery of Na^+ from root to shoot, and through tissue tolerance. Tissue tolerance is achieved by increasing sequestration of Na^+ into leaf vacuoles and accumulating K^+ and compatible solutes (such as proline, sucrose, glycine betaine, mannitol) in the cytosol and organelles to balance osmotic pressure of ions in vacuoles (Wang et al. 2005; Young-Pyo et al. 2007).

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Osmatic stress reduces the stomatal aperture restricting the exchange of CO_2 and O_2, leading to rapid ROS accumulation and oxidative stress (Roxas et al. 1997). ROS are partially reduced or activated derivatives of oxygen, including singlet oxygen ($^1O_2$), superoxide anion(O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$). They are highly reactive, toxic and may cause DNA or RNA damage, protein oxidation and lipid peroxidation (Bates et al. 1973). In general, the equilibrium between ROS production and removal by antioxidant defence components is strictly controlled. Under salinity stress, when CO_2 availability is restricted, this balance will be disturbed, leading to a remarkable increase in ROS concentration, namely an oxidative burst (Apostol et al. 1989). Apart from being highly reactive with numerous biomolecules and causing irreversible damage to plant cells, ROS can also activate a range of Na^+ and K^+ permeable ion channels (Demidchik et al. 2003) which disturb the cytosolic K^+/Na^+ ratio and lead to programmed cell death (PCD) (Demidchik et al. 2010; Shabala et al. 2007), as well as participate in signal transduction pathways and affect gene expression (Bradford 1976; Girotti 2001).

ROS detoxification in plants mainly involves two mechanisms: enzymatic and non-enzymatic scavenging mechanisms. Major non-enzymatic antioxidants include ascorbate, glutathione (GSH), a-tocopherol, carotenoids, while ROS scavenging enzymes are superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxidase (POD), Monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). In addition, osmolytes such as proline, mannitol, glycinebetaine, ectoine can be active in scavenging ROS and act through oxidative detoxification (Roxas et al. 1997; Shen et al. 1997).

Changes in antioxidant activities under salt stress were observed in both roots (Bandeoglu et al. 2004) and leaves (Ben Hamed et al. 2007). The lack of significant correlation between salinity stress tolerance and AO activity in roots (Chen et al. 2011; Maksimovic et al. 2013; Panda and Khan 2009) suggests that antioxidant ROS detoxification does not make a major contribution to salt tolerance in this tissue. As for leaves, the reported results are variable. While some researchers reported a positive association between antioxidant production in leaves and plant salinity tolerance (Jin et al. 2009; Kim et al. 2005; Moradi and Ismail 2007), others showed no or negative correlation between leaf AO activity and salinity stress tolerance (Abogadallah et al. 2010; Noreen et al. 2010; Parida and Jha 2010; Sabra et al. 2012). The possible mechanisms for such differences in AO activity remain unclear.

In this work we hypothesised that the possible answer to the above question may be a high tissue- and time-specificity of ROS production. Accordingly, this issue was addressed by elucidating kinetics of AO activity in leaves of various physiological age/position exposed to different periods of salinity stress. Then the selected "optimal treatments" were used to compare AO profiles of leaves of barley varieties contrasting in salt tolerance (three salt-tolerant and three salt-sensitive genotypes). We concluded that although salinity induced changes of leaf AO enzymes activities, they still cannot be used as biochemical indicators in breeding for salinity tolerance.

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

Six barley (*Hordeum vulgare* L.) varieties were used in this work. Among them, TX9425 (TX), YuYaoXiangTianErLeng (YYXT) and CM72 showed better tolerance to salt stress, while Naso Nijo (NN), Franklin and Gairdner were sensitive (Siripornadulsil et al. 2002). Seeds of all varieties were provided by the Tasmanian Barley Breeding Program. Seeds were sown in 2L-pots filled with potting mixture (Pang et al. 2004). Six pots, each contained a single variety with five plants, were placed in a 40-L bin, representing one replication of a treatment. Three replications were applied for all the treatments. Seedlings were grown in a glasshouse with controlled temperature (25 ± 2 °C) under natural sunlight at Mt Pleasant Laboratories in Launceston.

3.2.2 Treatments and sample collection

Salt stress was started at the four leaf stage (15 d-old seedlings). Salt solution (240 mM NaCl) was used to wash through the pots several times until the solution drained out from the pots had consistent salt concentration. The treatment was repeated every three days. Two contrasting varieties, TX and NN were used for time- and tissue-dependence experiments (so-called "Experiment I"). The second and third leaves from the bottom were collected for enzyme measurements after 1, 2, 5, 10 days of salt treatment. In Experiment II, the third leaves from the bottom of six varieties were collected ten days after NaCl treatment for the measurement of antioxidant enzyme activities, MDA and proline content. Chlorophyll and Na^+, K^+ contents were measured in the first fully expanded leaves.

3.2.3 Grain yield and plant survival under salt stress

Grain yield: Varieties were sown in large tanks (160 by 120 by 60 cm) filled with potting mixture and located in a glasshouse. Each variety contained 25-30 plants in a 70 cm - long row, with 15 cm gap between rows. After germination, two replications were treated with 240 mM NaCl and the other two replications were used as a control. All containers were linked with a drainage system connected to the bottom of each container and an application system across the top of each container. A 160-L sump was placed on the floor 1.5 m below the top of the containers and connected to a pump capable of lifting water from the sump to the top of the containers. After germination, 240 mM NaCl solution was pumped from the sump through the application system to the top of the containers which were used for salt treatment. The drainage system was kept closed until solution accumulated to a depth of 10 mm over the surface of potting mixture. The pump was then switched off and the containers were allowed to remain soaked with the solution for 10–15 min. The drainage system was then reopened and the salt solution returned to the sump. More salt was added to adjust the solution to 240 mM. This procedure was repeated several times until the solution drained from the containers reached a consistent salt concentration (Siripornadulsil et al. 2002). This process was repeated at weekly intervals. After maturity, grain yield of both salt treated and controls were recorded.

Plant survival: plant survival rate has been used by many researchers as a reliable indication of plant salt tolerance. A higher salt stress (320 mM NaCl) was also applied in this experiment. Most of the very sensitive varieties will not be able to survive to maturity under this concentration. A similar treatment system as described above was applied but with no controls. Plant survival was scored according to leaf chlorosis and plant healthiness (0 = all dead and 10 = no damage).

3.2.4 Measurement of Leaf chlorophyll content

Leaf chlorophyll content was tested in first fully expanded leaves using SPAD-502 chlorophyll meter (Minolta, Tokyo, Japan).

3.2.5 Determination of antioxidant enzyme activity and protein contents

For extraction of antioxidant enzymes, 0.5 g of leaf samples was homogenized in mortars with 7 mL phosphate buffer solution (PBS). Fresh leaf samples were used in both

experiments. The extraction buffer was 50 mM PBS (pH 7.8) containing 0.1 mM EDTA and 2% PVP. The homogenates were centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatants were collected (stored in 4°C) to evaluate the enzyme activities which were determined by a spectrophotometer (Genesys10S UV-VIS).

SOD activity was evaluated by the ability to inhibit photo-reduction of nitroblue tetrazolium (NBT) (Beyer and Fridovich 1987). CAT activity was determined by monitoring the disappearance rate of H_2O_2 at 240 nm according to the method of Aebi (1984). APX activity was assayed by following the rate of H_2O_2-dependent oxidation of ascorbic acid (AsA) according to the method of Nakano and Asada (1981). POD activity was assessed by recording the increased absorbance at 470 nm due to the oxidation of guaiacol according to Chance and Maehly (1955).

Protein content was determined according to the method described by Bradford (1976) at 595 nm using bovine serum albumin as a standard.

3.2.6 Determination of Na^+ and K^+ in leaves

To determine the sodium and potassium ions content, leaf sap of first fully expanded leaves were extracted and centrifuged at 5000 rpm for 10 min essentially as described elsewhere (Cuin et al. 2009). The supernatants were collected to evaluate Na^+ and K^+ content using a flame photometer.

3.2.7 Estimation of lipid peroxidation and proline content

The level of lipid peroxidation was determined in terms of MDA content, a product of lipid peroxidation, following a modified method of Heath and Packer (1968).

Proline content was estimated according to the method of Bates et al. (1973) and Sayed et al. (2012a). Leaf samples were collected and ground to fine power. Proline content was determined by a standard curve from known concentrations of L-proline.

Data analysis was conducted in Excel.

3.3 Results

3.3.1 Growth and agronomical characters of barley varieties

Salinity significantly impacted plant growth and yield. The yield of all the varieties showed a significant ($P < 0.05$) decrease, being more severe for sensitive varieties under 240 mM NaCl stress (Figure 3.1A). Tolerant varieties (TX, CM72, YYXT) had greater plant survival under high salt stress than sensitive ones (NN, Gairdner, Franklin) (Figure 3.1B). Figure 3.1C shows a typical difference between a tolerant variety (TX) and a sensitive one (NN). After 10 days of 320 mM NaCl treatment starting from germination, no obvious symptom of salt stress was noticed in TX while numerous yellow or dead leaves were found in NN. Plant survival under high salt stress was consistent with relative yields at lower salt stress (240 mM NaCl).

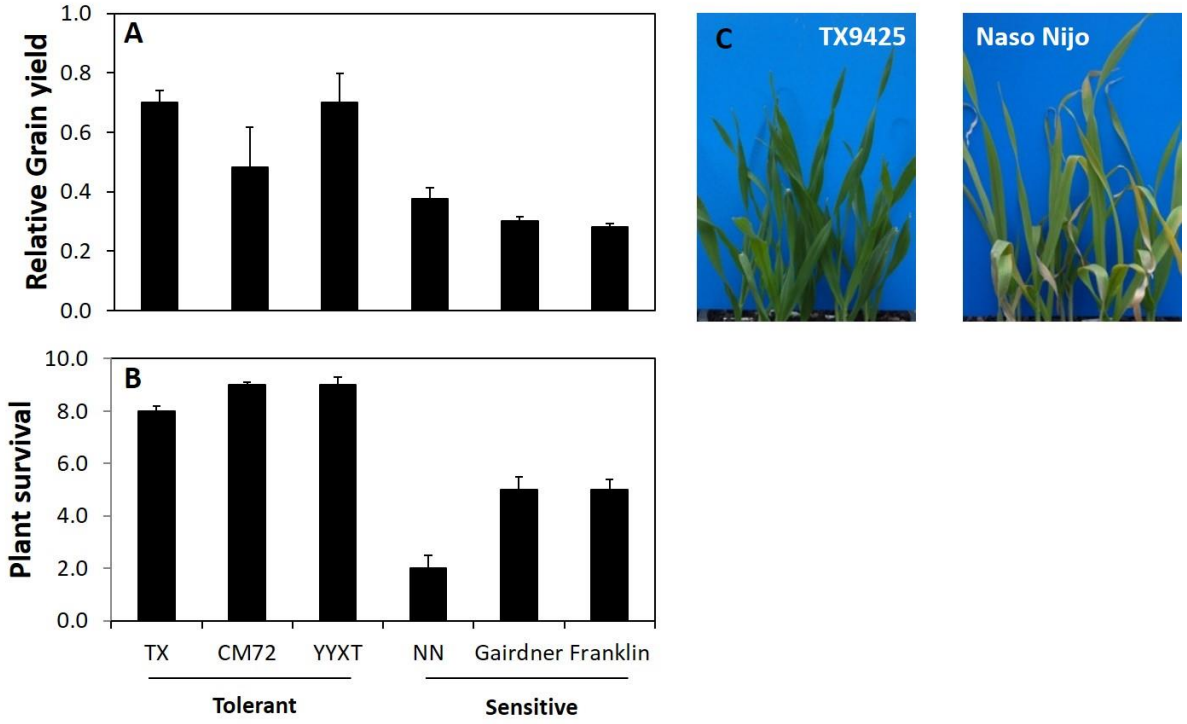

Figure 3.1 Growth, survival and grain yield of barley varieties under salt stress. A, Relative grain yield of six barley varieties under 240 mM NaCl stress; B, Plant survival score of six barley varieties under 320 mM NaCl stress (0 = all dead; 10 = no obvious symptom); C, Plants treated with 320mM NaCl after germination growth with TX9425 showing much better salt tolerance than Naso Nijo.

Table 3.1 ANOVA analysis of antioxidant enzyme activities (SOD, CAT, APX, POD) in TX and NN after 1, 2, 5, 10 days of 240 mM NaCl treatment. V: variety; T: treatment; L: leaf age; D1-10: Days after salt treatment; *: P < 0.05; **: P < 0.01.

	SOD				CAT				APX				POD			
Source of variance	D1 F	D2 F	D5 F	D10 F	D1 F	D2 F	D5 F	D10 F	D1 F	D2 F	D5 F	D10 F	D1 F	D2 F	D5 F	D10 F
V	1.9	5.2	0.2	8.5*	24.6**	5.6*	4.0	5.0*	1.0	0.0	9.2**	0.3	1.9	0.7	1.1	0.8
T	5.3	4.1	21.0**	85.9**	1.1	0.6	6.7*	0.2	6.0*	4.8	8.4*	2.4	1.1	11.9*	0.5	5.8*
L	1.3	0.9	0.2	0.3	4.1	9.9*	24.7**	0.7	0.2	1.3	7.4*	0.3	24.4**	114.3**	22.0**	43.1**
V-T	0.0	0.7	0.1	2.7	0.3	6.8*	1.0	5.9*	1.6	0.1	0.9	5.9*	0.2	7.2*	0.8	7.8*
V-L	0.2	0.1	0.0	0.1	1.4	0.0	4.6	5.8*	0.8	41.9**	2.7	0.3	0.0	0.1	2.1	9.1**
T-L	0.0	0.1	0.1	4.8*	3.8	4.1	0.1	0.2	1.3	0.0	0.0	4.1	0.8	8.0*	0.5	1.5
V-T-L	1.0	1.9	0.5	0.1	6.3*	0.8	0.3	0.0	0.7	0.4	0.3	0.2	0.1	0.7	0.1	0.1

3.3.2 Activities of antioxidant enzymes

In Experiment I, two contrasting varieties - TX (tolerant) and NN (sensitive) - were selected to investigate the effect of leaf age and duration of salt exposure on antioxidant enzyme activity. After 1, 2, 5, 10 days of 240 mM NaCl treatment, enzyme activities in 2nd and 3rd leaves of both varieties were measured (Table 3.1). SOD activity in both 2nd and 3rd leaves increased in both varieties after salt treatment (Figure 3.2A, B). No significant difference was found for either CAT or APX activity in the two leaves of both varieties under salt treatment (Figure 3.2C-F). Under salt treatment, TX did not show significant changes in POD activity while POD activity was enhanced in NN, especially 2 and 10 days after the treatment (Figure 3.2G, H). Younger leaves showed much lower POD activity but the changes caused by salt treatment were similar in both varieties. As shown in Figure 3.2, treatment times had little effect on changes in the activity of different enzymes. In general, slightly larger difference was found between varieties at the 10th day after treatment. For example, POD activity of NN increased in both younger and older leaves after salt treatment, while there was no significant change in POD activity in the leaves of TX. Thus, further measurements (Experiment II) of

different enzyme activities on other varieties were conducted using the third leaves and after 10 days of salt treatment.

Similar to the results in Experiment I, SOD activity of all varieties but Franklin increased under salt stress, with no obvious correlation with salt tolerance of the varieties (Figure 3.3A). Under salt stress, CAT activities showed trends of increase (relative to control) in tolerant varieties and decrease in sensitive varieties (Figure 3.3B). APX activities of all varieties were enhanced with no obvious differences between tolerant and sensitive varieties (Figure 3.3C). POD activity of all tolerant varieties showed a significant increase after salt treatment with only one sensitive variety (NN) showing significant increase in POD activity (Figure 3.3D). Again, no significant correlation between POD activity and plant salt stress tolerance was found (Table 3.2).

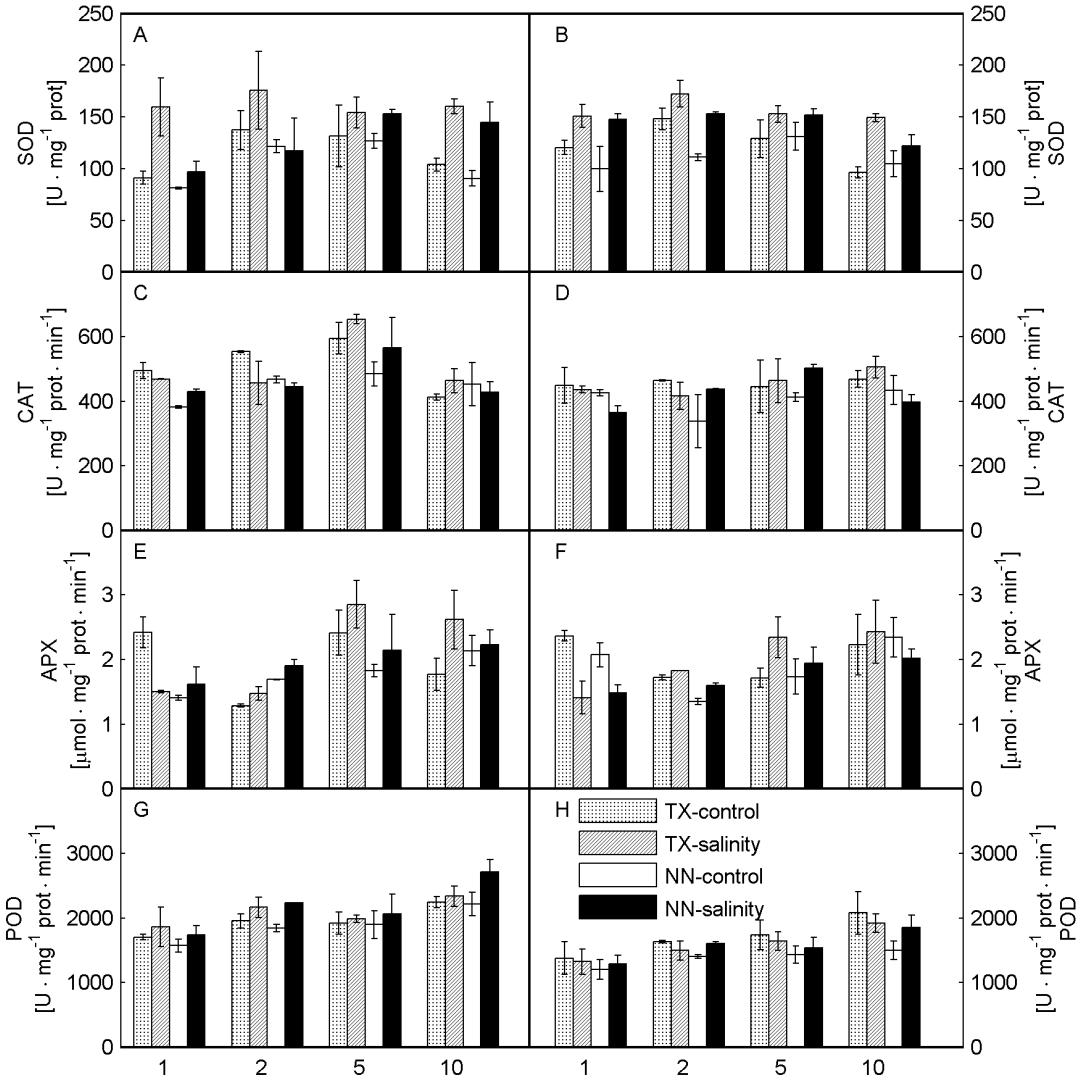

Figure 3.2 Antioxidant enzyme activities of second leaves (A, C, E, G) and third leaves (B, D, F, H) from the bottom in TX9425 (TX) and Naso Nijo (NN) at 1, 2, 5, 10 days after 240 mM NaCl treatment. Older leaves (second from bottom) had higher enzyme activities (especially for POD). The differences among varieties, treatment times were more pronounced in the third leaves at 10th day after salt treatment. A-B, SOD activity; C-D, CAT activity; E-F, APX activity; G-H, POD activity. Mean ± SE (n = 3, each sample contained leaves from at least 3 plants)

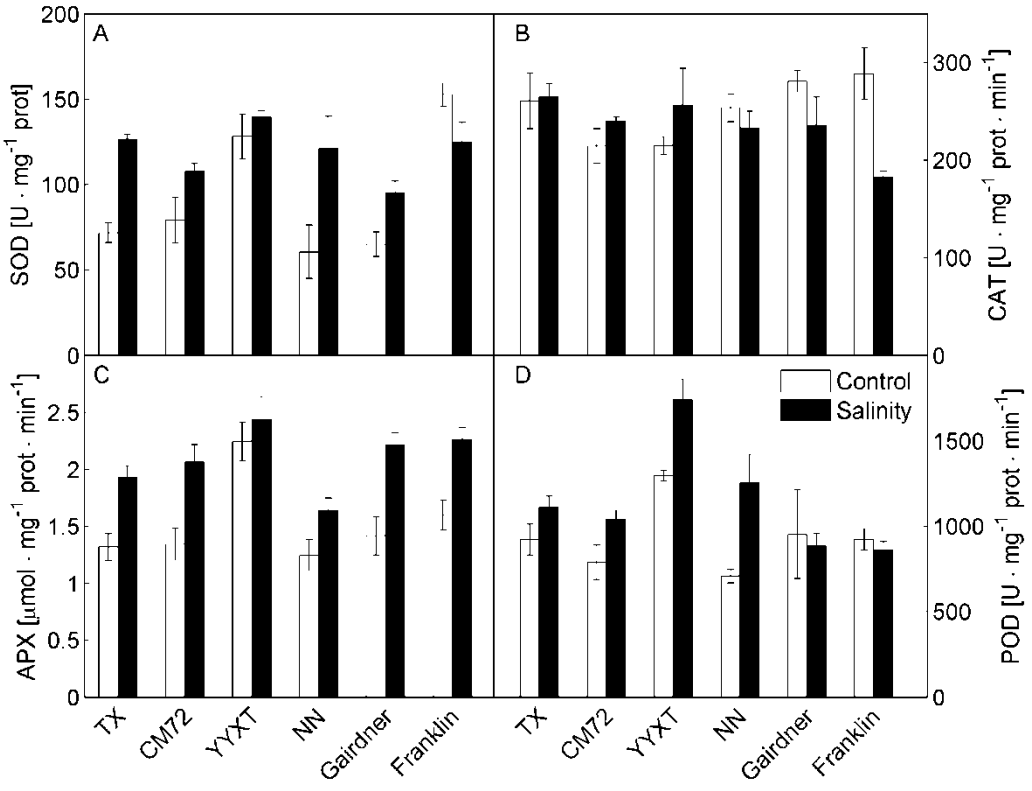

Figure 3.3 Antioxidant enzyme activities of third leaves (at four leaves stage) in six barley varieties contrasting in salt tolerance (Tolerant: TX, CM72, YYXT; Sensitive: NN, Gairdner, Franklin) after 10 days 240mM NaCl treatment. A, SOD activity; B, CAT activity; C, APX activity; D, POD activity. No evident differences of enzyme activities were observed between tolerant and sensitive varieties. Mean ± SE (n = 3, each sample contained leaves from at least 3 plants)

3.3.3 Na^+ and K^+ content in leaves

Na^+ exclusion and K^+ retention are considered to be key mechanisms for plant tolerance to salinity (Shabala and Cuin 2008). In Experiment I, Na^+ content in leaves of both varieties increased significantly after salt treatment. The longer the treatment, the higher the Na^+ content (Figure 3.4A). Na^+/K^+ ratios showed a trend similar to one for Na^+ content (Figure 3.4C). The tolerant variety showed consistently lower Na^+ contents and lower Na^+/K^+ ratios. No significant changes were found in the K^+ content of all except NN on the 10th day (not shown in the figure).

In Experiment II, higher Na^+ contents and Na^+/K^+ ratios could be seen in salt sensitive genotypes except for Gairdner (Figure 3.4B, D). Both traits had negative correlation with plant survival (Table 3.2).

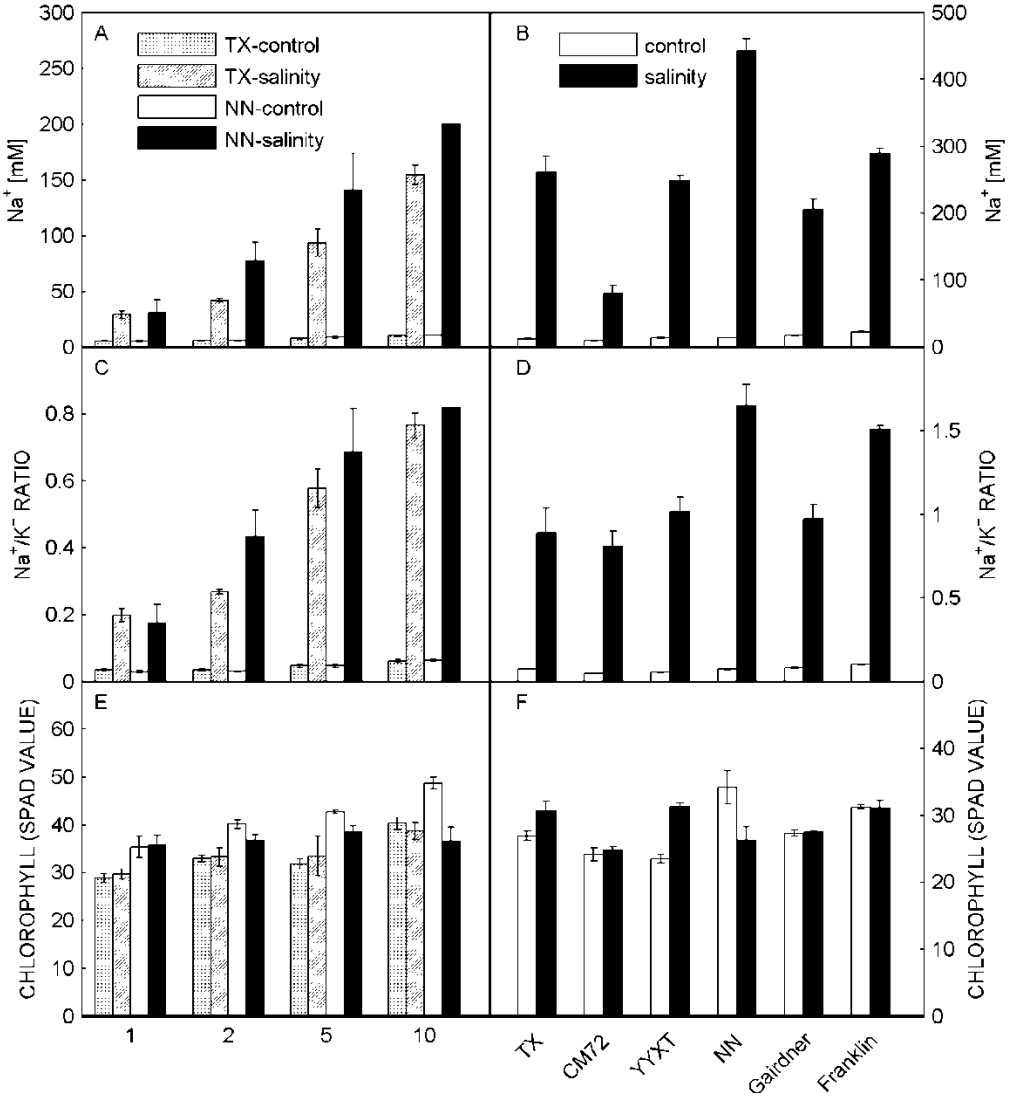

Figure 3.4 Na^+, Na^+/K^+ and chlorophyll content (in 1st fully expanded leaves). A, Na^+ content, C, Na^+/K^+ ratio and E, chlorophyll content (SPAD value) of TX and NN at 1, 2, 5, 10 days after 240 mM NaCl treatment; B, Na^+ content, D, Na^+/K^+ ratio and F, chlorophyll content of six barley varieties contrasting in salt tolerance after 10 days 240mM NaCl treatment. Salinity induced changes of Na^+, Na^+/K^+ and chlorophyll content (A, C, E), and obvious difference ($p<0.05$) between tolerant and sensitive varieties (B, D, F). Mean ± SE (n = 5)

Table 3.2 Correlations between antioxidant enzyme activities (SOD, CAT, APX, POD), MDA, Proline, Na^+, K^+, Na^+/K^+ ratio, chlorophyll content and grain yield, plant survival in six barley varieties contrasting in salt tolerance. Higher plant survival score meant better surviving ability (The plant survival scores: 0 = all dead; 10 = no obvious symptom). *: $P < 0.05$.

	SOD	CAT	APX	POD	MDA	Proline	Na	K	Na/K	Chl	Grain yield	Plant Survival
SOD	1											
CAT	0.231	1										
APX	0.176	-0.384	1									
POD	0.634	0.475	-0.445	1								
MDA	0.599	0.292	-0.346	0.783	1							
Proline	0.608	-0.325	-0.131	0.656	0.596	1						
Na	0.387	-0.355	-0.436	0.481	0.554	0.870*	1					
K	0.660	-0.020	-0.124	0.272	0.623	0.468	0.631	1				
Na/K	0.047	-0.616	-0.283	0.322	0.309	0.819*	0.844	0.173	1			
Chl	-0.478	0.544	-0.428	-0.326	-0.202	-0.792	-0.456	-0.082	-0.637	1		
Grain yield	0.136	0.804	-0.467	0.256	0.451	-0.359	-0.164	0.358	-0.546	0.740	1	
Plant Survival	-0.363	0.674	-0.093	-0.239	-0.127	-0.862*	-0.766	-0.277	-0.839*	0.844*	0.723	1

3.3.4 Chlorophyll content in leaves

Chlorophyll contents of the first fully expanded leaves were measured in TX and NN at 1, 2, 5 and 10 days after salt treatment. Salt treatment showed no significant effects on chlorophyll contents of TX genotype while in NN plants it showed a continuous decrease with extended treatment times (Figure 3.4E). Similar results were found for other tolerant varieties which showed no changes or even an increase in leaf chlorophyll content. However, chlorophyll contents of two other sensitive varieties were not affected by salt treatment (Figure 3.4F). Positive correlations were found between relative chlorophyll content and plant survival or relative grain yield (Table 3.2).

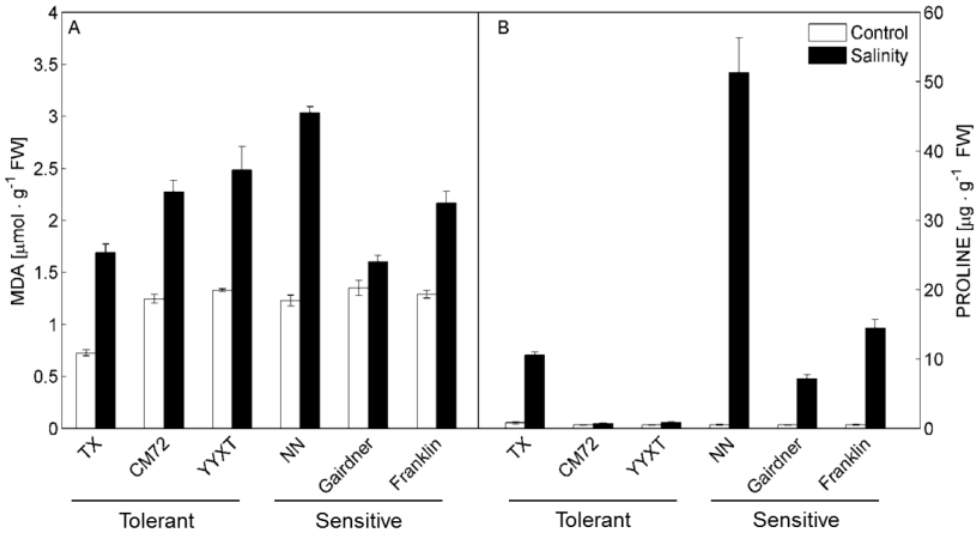

Figure 3.5 MDA and proline content (in 3rd leaves from bottom) of six barley varieties at 10 days after 240mM NaCl treatment. A, MDA content; B, proline content. Under salinity stress, MDA increased in all while proline showed significant difference ($p < 0.05$) between tolerant and sensitive varieties. Mean ± SE (n = 3, each sample contained leaves from at least 3 plants)

3.3.5 Lipid peroxidation

As a product of lipid peroxidation, MDA is generally used as an indicator of levels of lipid peroxidation. As can be seen from Figure 3.5A, MDA contents increased in all varieties 10 days after salt treatment. No obvious patterns were revealed for MDA levels between salt tolerant and sensitive genotypes (Figure 3.5A), reflected by the results of correlation analysis (Table 3.2).

3.3.6 Proline content

Plants need compatible solutes such as proline in cytosol and organelles to balance the osmotic pressure of ions in vacuoles caused by salinity. Higher proline contents were induced under salt stress in all sensitive varieties and one of the tolerant varieties TX, while no obvious changes were found in other two tolerant varieties CM72 and YYXT (Figure 3.5B). Proline content under salt stress exhibited significant negative correlation with plant survival (Table 3.2).

3.4 Discussion

3.4.1 Leaf antioxidant enzyme activities do not correlate with salinity tolerance

Contrasting salt concentrations has been widely used to investigate the change of various antioxidants under salt stress in different species (Ben Amor et al. 2006; Parida and Jha 2010; Sabra et al. 2012; Sergio et al. 2012). However, there were very few reports on influences of treatment times and leaf ages (e.g. younger and older leaves) on antioxidants activities, which may be one of the reasons for the inconsistency of results from different reports. Salinity-specific induction of SOD isoforms in barley was reported by Kim et al. (2005). The differences in enzyme activities came from different experimental conditions such as exposure time, salt level and genotypes (Jin et al. 2009; Kim et al. 2005). Growing seasons also affected antioxidant enzyme activity since plants grew slower in winter and photosynthesis rate differed if plants were grown in the field or in sites without consistent light and temperature conditions (Ben Ahmed et al. 2009). ROS are reduced or activated derivatives of oxygen produced by aerobic metabolism such as photosynthesis and respiration, and they are highly reactive (Bates et al. 1973). Since ROS production and antioxidant enzymes are highly unstable and time-dependent, higher antioxidant activities at one particular “snapshot” (a measurement at one specific time) do not represent higher plant salt tolerance. In this work, two varieties differing in their salt tolerance were used to investigate changes in leaf antioxidant enzyme activity in different leaves after different times of salt treatment. Salt stress caused significant changes in activities of most of the enzymes but treatment times showed little effect on enzyme activities (Figure 3.2). Leaf ages (younger and older) had significant effects on POD activity, with the activities from younger leaves being much lower.

Oxidative stress can cause major damage to plants, thus the detoxification ability of plants becomes very important. However, it is still not clear whether higher levels antioxidants represent greater plant salt tolerance. A large number of researchers proved positive association between antioxidant production and salt tolerance of plants including barley (Jin et al. 2009), rice (Moradi and Ismail 2007), *Arabidopsis* (Attia et al. 2008) and halophyte *Cakile maritime* (Ben Amor et al. 2006). Transgenic plants overexpressing antioxidant genes also exhibited better salt tolerance (Eltayeb et al. 2007; Sreenivasulu et al. 2004; Ushimaru et al. 2006; Wang et al. 2004). In contrast, there are also numerous reports that activities of antioxidants declined under salt stress (Abogadallah et al. 2010; Hafsi et al. 2010; Noreen et

al. 2010; Yang et al. 2010). Munns and Tester (2008) suggested that genetic differences of salinity tolerance does not necessarily attribute to differences of ROS detoxifying ability. A similar conclusion was made by Maksimovic et al. (2013), pointing out that higher antioxidant activities at one particular time did not represent higher salt tolerance since higher antioxidant activities were observed in sensitive plants and no correlation between SOD activity and salt tolerance was found under a large scale screening. In this experiment, salt treatment caused a significant increase in SOD activity of all varieties but Franklin (Figure 3.3A). APX activities of almost all varieties increased and no significant decreases in POD activity were found in any of six varieties (Figure 3.3C, D). Overall, the activities of the three antioxidants (SOD, APX and POD) showed no correlations with salt tolerance (Table 3.2) and thus cannot be used as selection criteria for salt tolerance. Interestingly, CAT activities of all tolerant varieties tended to increase while those of all sensitive varieties tended to decrease under salt stress (Figure 3.3B). However, the difference was small and could not reliably be used in selecting salt tolerant varieties.

3.4.2 Tolerant varieties have lower Na^+/K^+ ratio and higher chlorophyll content in leaves

Higher K^+/Na^+ ratio (resulting from either better K^+ retention or Na^+ exclusion, or both) is considered as a key determinant of salt tolerance (Chen et al. 2007b; Chen et al. 2007c; Shabala and Cuin 2008). Higher Na^+ content and Na^+/ K^+ ratio in sensitive varieties were observed in this experiment (Figure 3.4B, D); both showed highly significant negative correlations with plant survival (Table 3.2). These results indicated that tolerant genotypes used in this study had a better ability for Na^+ exclusion, leading to lower Na^+ contents and lower Na^+/ K^+ ratios in leaves which was consistent with previous reports (Abraham and Dhar 2010; Chen et al. 2007c; Garthwaite et al. 2005; Munns and James 2003). Leaf chlorosis was considered as an adaptation by retaining internal water for transpiration demands various stresses, for example drought stress (Champoux et al. 1995). Positive correlations between relative chlorophyll contents under salt stress and plant survival were also found in this study (Figure 3.4E, F; Table 3.2). The relatively higher chlorophyll contents in salt tolerant varieties was partly due to less leaf chlorosis under salinity of the tolerant genotypes (El-Tayeb 2005; Munns and James 2003; Panda and Khan 2009; Wu et al. 2013). Leaf chlorosis or leaf senescence are recognised as examples of PCD which could be triggered under salinity due to Na^+ accumulation or K^+ loss (Shabala 2009; Shabala et al. 2007).

3.4.3 Salt-stress increases lipid peroxidation in all varieties

ROS scavenging mechanisms mainly include SOD, CAT, ascorbate-glutathione cycle and GPX cycle. In the ascorbate-glutathione cycle, APX detoxifies H_2O_2 to H_2O and simultaneously ascorbate is oxidated to MDA. After that, MDA could be reduced to ascorbate by MDAR with the help of NADPH (Bradford 1976). Therefore, MDA is usually deemed as an indicator of lipid peroxidation. In this experiment, MDA content increased under salt stress for all varieties (Figure 3.5A), which is due to more ROS production under salt stress (Abogadallah et al. 2010; Chen et al. 2011; El-Tayeb 2005). No obvious correlation was found between MDA contents and plant survival or grain yield under salt stress (Figure 3.5A; Table 3.2), thus the production of MDA may not be used as a physiological marker for evaluating the extent of plant salt tolerance.

3.4.4 Proline accumulates more in sensitive varieties and positively correlates with Na^+ content and Na^+/K^+ ratio in leaves

Apart from being an osmolyte to balance osmotic pressure in cells, proline also acts as a ROS scavenger and plays an important role in reducing oxidative stress induced by osmotic stress (Cuin and Shabala 2007b; Hong et al. 2000; Kaul et al. 2008; Matysik et al. 2002; Szekely et al. 2008). During stress, the reduced rate of the Calvin cycle causes insufficient electron acceptor $NADP^+$ and leads to ROS accumulation in green leaves (Chaves et al. 2009). Proline biosynthesis in chloroplast maintains low NADPH: $NADP^+$ ratios to sustain electron flow, thus reducing the extent of photoinhibition and ROS production. Proline degradation in mitochondrion provides electrons for the respiratory chain (Hare and Cress 1997; Kishor et al. 2005). Therefore, proline catabolism is important for regulating cellular ROS balance, and the balance between proline biosynthesis and degradation is also critical (Szabados and Savoure 2010; Verbruggen and Hermans 2008). Salinity-induced increase in proline accumulation has been reported in many publications (Gorham et al. 1985; Ueda et al. 2007). However, the correlation between proline accumulation and salinity tolerance in plants is still obscure and the gain or loss of function or exogenously applied strategies did not bring any clear answers (Ashraf and Foolad 2007; Szabados and Savoure 2010; Verbruggen and Hermans 2008). Proline accumulation has been reported under different environmental stresses such as drought (Choudhary et al. 2005), low temperature (Naidu et al. 1991), oxidative stess (Yang et al. 2009b) and heavy metals (Schat et al. 1997; Siripornadulsil et al. 2002). In this work, the higher proline content induced by salinity stress was observed in all

sensitive barley varieties. In contrast, only one tolerant variety (TX) showed significant increase in proline content under salt stress (Figure 3.5B). Proline contents showed a significant positive correlation with Na^+ contents and Na^+/K^+ ratios and a negative correlation with plant survival (Table 3.2), consistent with most of previous reports (Hong et al. 2000; Hoque et al. 2008; Hoque et al. 2007; Khedr et al. 2003; Murakeozy et al. 2003; Taji et al. 2004). Thus, proline Proline accumulation has been treated as an indication of salt injury in many studies (Liu and Zhu 1997; Lutts et al. 1999; Moradi and Ismail 2007). However, proline accumulation can only be an indicator of Na^+ concentration in our study, possibly due to its function as compatible solutes to balance the osmotic pressure of ion in vacuole. Since compatible solutes synthesis is energetically costly (e.g. 41 moles of ATP is required to produce one mole of proline; 50 for glycine betaine), they help plants survive and recover from salinity stress at the expense of growth (Munns and Tester 2008; Raven 1985). This statement was supported by our study that proline accumulation showed relatively higher correlation with plant survival than with relative grain yield.

In conclusion, changes in the level of antioxidant enzyme activity and lipid peroxidation were induced by salt stress and activities of leaf antioxidant enzymes were influenced by leaf age, salt concentration, time of treatment, and genotype. However, no significant correlation between plant salt tolerance and antioxidant enzyme activity or MDA content was observed. Chlorophyll and proline contents and Na^+/K^+ ratio may be used as possible criteria for selecting salt tolerant varieties.

Chapter 4 Using QTL mapping to investigate the relationships between abiotic stress tolerance (drought and salinity) and agronomic and physiological traits

4.1 Background

Drought and salinity are two major abiotic stresses that severely limit agricultural production worldwide. The severity and occurrence of both drought and salinity stresses is going to increase as a result of global environmental changes, with a major implication for food supply (Shabala 2013; Tester and Langridge 2010). On the other hand, increasing world population requires increase in food production by more than 70% by 2050 (FAO 2011). One of the sustainable and economical solutions to achieve this goal is developing drought and salt-tolerant crops (Ashraf 2009). However, very slow progress has been made in improving tolerance or developing tolerant cultivars due physiological and genetic (quantitative inheritance) complexity of tolerance traits. Also, high variability of the field environments and low efficiency of selection methods further handicapped the progress. Most researchers agree that it is highly unlikely that tolerance to these stresses may be improved by a manipulating with expression level (function) of merely one gene. More likely, we should brace ourselves for a painstakingly slow pyramiding of useful traits. Taking salinity stress tolerance as an example, vacuolar Na^+ sequestration mediated by NHX Na^+/H^+ exchanger (Apse et al. 1999) could be not possible without sufficient activity of tonoplast H^+-pump to energize this process (Shabala 2013). Moreover, this sequestration will become a futile cycle if Na^+ back-leak from vacuole via Na^+-permeable fast (FV)- and slow (SV)- vacuolar channels is not prevented (Bonales-Alatorre et al. 2013a; Bonales-Alatorre et al. 2013b). Given that the molecular identity of some of this transport systems (e.g. FV channels) is yet to be revealed, transgenic approach to such pyramiding remains highly challenging.

In addition to the "biological" complexity of the salinity- and drought- tolerance issue, a social aspect of the problem should be not ignored. The generally negative public perception of GMO world-wide casts serious doubt over the prospects of tackling this issue by a broad use of transgenic crops. The recently imposed total ban on the use of GM-crops in Tasmania

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is a good illustration of this fact. During a recent Barley Technical Symposium in Adelaide in 2013, representatives of major brewing companies were unanimous in their estimation that, in light of the above, transgenic malting barley varieties are unlikely to be accepted by the Industry in a foreseeable future. This calls for a renewed interest in using more traditional and publically accepted technologies such as Marker-Assisted Selection (MAS) or the newly developed genome editing technology.

MAS technology implies the use of a set of markers which are closely linked with the target gene(s) for an indirect selection of a specific traits without phenotyping the traits. While a great progress has been achieved in using MAS approach for crop breeding for a range of stresses where the tolerance is conferred by one or two major genes, the progress was more modest when it comes to salinity or drought tolerance. Numerous physiological and morphological traits were used as indirect selection criteria for both salinity and drought tolerance. Leaf wilting, relative water content (RWC) and proline contents are among the most frequently used for drought tolerance (Condon et al. 2002; Richards et al. 2002; Teulat et al. 2003). Physiological and biochemical responses used as selection criteria for salinity tolerance include seed germination under stress conditions, relative water content, wet and dry weight of roots and shoots, chlorophyll content, shoot sodium content, plant survival as well as tissue proline and carbohydrate content (Chen et al. 2005; Shavrukov et al. 2010; Xu et al. 2012).

Proline is a widely distributed osmolyte which protects plants against drought and salinity (Bohnert et al. 1995). It is mainly synthesized from glutamate by two enzymes: pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) (Szabados and Savoure 2010). Apart from acting as an osmolyte to balance osmotic pressure in cells, proline also plays important roles in regulating cellular reactive oxygen species (ROS) balance (Hong et al. 2000; Yamaguchi and Blumwald 2005), cell signalling and plant development like rapid cell division, floral transition and embryo development (Mytinova et al. 2010). Proline was also shown to be able to affect intracellular ionic homeostasis by controlling ion transport across cellular membranes (Cuin and Shabala 2005, 2007a). Proline level increased dramatically in plants under both drought (Choudhary et al. 2005) and salinity (Yoshiba et al. 1995) conditions, and it was repeatedly suggested that using high proline levels as a biochemical marker may benefit stress breeding programs (reviewed in (Ashraf et al. 2008)). However, higher proline levels were also found in drought-hypersensitive (Hanson et al. 1979;

Singh et al. 1972) and salinity-susceptible genotypes (Ashraf and Foolad 2013; Moradi and Ismail 2007), and the causal relationship between proline accumulation and stress tolerance in plants is not that straight forward as initially thought.

In a natural environment, drought and salinity stress are often combined (Katerji et al. 2009). Both drought and salt stress trigger cellular dehydration and cause osmotic stress which then lead to cytosolic and vacuolar volume reduction (Munns 2002; Zhu 2002). Abiotic stress such as cold, drought and salt stress are controlled by many common and conserved regulatory pathways (Rabbani et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2006). Drought tolerance QTL influenced growth under salt stress by reducing salt uptake (Sharma et al. 2011), indicating that some QTL/genes may have pleiotropic effects on multi-stress tolerance.

Both drought and salinity tolerance are quantitatively inherited and controlled by several genetic loci. While many QTLs being reported for drought (Baum et al. 2003; Kalladan et al. 2013; Sayed et al. 2012b; Teulat et al. 2001a) and salinity tolerance (Rivandi et al. 2011; Shavrukov et al. 2010; Xu et al. 2012; Zhou et al. 2012), very few of the linked markers have been successfully used in breeding programs due to the relatively lower heritability of the QTL and other factors affecting the gene expression. The success of using physiological traits as indirect selecting criteria for both drought and salinity tolerance relies on the true correlations between these traits and the tolerance. Most studies used very few varieties to study the relationships between drought/salinity tolerance and different agronomic/physiological traits or simply mapping QTLs for different traits under drought or salinity stress (Kalladan et al. 2013; Teulat et al. 2001a; Teulat et al. 2003; von Korff et al. 2008), which may not necessarily reflect the tolerance genes. This issue was overcome in this work that a doubled haploid (DH) population was used: 1) to investigate the linkage between various agronomic and physiological traits and drought and salinity tolerance, and 2) to identify QTLs controlling tolerance to these two stresses in barley.

4.2 Materials and methods

4.2.1 Plant material

A total of 74 F_1-derived doubled-haploid (DH) lines generated from a cross between TX9425 (drought and salinity tolerant) and Franklin (drought and salinity sensitive) were used in this study. TX9425 is a Chinese landrace two-rowed barley variety which also exhibited some

particular agronomic traits (Wang et al. 2010) and disease resistance (Li et al. 2009). Franklin is an Australian two-rowed malting barley and it was regarded as salinity sensitive variety (Chen et al. 2008; Zhou et al. 2012).

4.2.2 Evaluation of drought tolerance and relevant physiological traits

Three separate experiments were conducted for evaluating drought tolerances; each experiment was repeated three times.

Experiment I and II: five seeds of parental varieties and DH lines were sown in big containers (1.6 m x 2.5 m x 0.6 m) filled with a pine bark/loam-based potting mix with premixed slow release fertiliser. The containers were located in a glasshouse at the Mt Pleasant Laboratories in Launceston, Tasmania. Trials were conducted in 2012/13 and 2013/14 growing seasons. The trials were kept watered in early growth stage. At early tillering stage, the watering stopped in half of the containers, and the latter were left drying. When the most susceptible lines showed severe symptoms of wilting (approximately four weeks after drought treatment, Figure 4.1), the scoring of wilting was conducted in Exp. I and II (0= drought tolerant with no damage and 10= drought sensitive with pronounced leaf wilting) and the second fully expanded leaves (Exp. II) were sampled for the evaluation of proline content.

Figure 4.1 Drought tolerance of parents and few DH lines. A: Experiment I and II (left: tolerant parent TX9425; middle: sensitive parent Franklin; right: one medium tolerant DH line); B: Experiment III (left: sensitive DH line; right: tolerant DH line).

Experiment III: each parent varieties or DH lines were sown in 2-L pots filled with potting mixture. All the pots were placed in six different trays, each contained a whole replication. The water level was kept 2-3 cm high in the tray. Half of the trays were kept dry starting from

the early stage of tillering. Similar to Exp. I and II, when the most susceptible lines showed severe symptoms of wilting, the scoring of wilting was conducted and the second fully expanded leaves were sampled for the evaluation of proline content. The first and second fully extended leaves from different plants were sampled for measuring moisture content.

4.2.3 Evaluation of salinity tolerance and relevant physiological traits

Seeds of parental varieties and the DH lines were sown in big plastic containers (1.6m x 0.8m x 0.6m) filled with a pine bark/loam based potting mixture with premixed slow release fertiliser. Each genotype comprised of three replicates, each of five seeds. Controls were omitted in this case since it has been showed in our earlier report that different varieties or DH lines grown in the same potting mixture but with no salt added showed no apparent symptoms of leaf chlorosis or dead leaves (Zhou et al. 2012). The salt treatment was similar to previously described (Xu et al. 2012; Zhou et al. 2012). Salt stress was started at the three-leaf stage. A solution containing 320 mM NaCl was used to wash through the tanks several times until the solution drained out from the tanks had a consistent salt concentration. The treatment was repeated every three days. When the most susceptible lines showed severe symptoms, salt tolerance was assessed by combining scores for leaf chlorosis and plant survival when most of the DH lines reached booting stage (0= no damage and 10= all dead) (Xu et al. 2012). The second leaves of the DH lines were collected for proline assay and top two leaves from different plants were collected for measuring Na^+ contents.

4.2.4 Measurement of Na^+ content in leaves and relative moisture content

Fresh leaves were weighed soon after collection. The samples were dried in a 60ºC oven for two days and dry weights were then recorded. Moisture content calculated from fresh weight and dry weight of the samples. For the Na content, leaf sap were extracted and centrifuged at 5000 rpm for 10 min as described elsewhere (Munns 2007). The supernatants were collected to evaluate Na^+ content using a flame photometer.

4.2.5 Measurement of proline content

Proline content was estimated according to the method of Mittler (Mittler 2002) and Sayed (Sayed et al. 2012b). Leaf samples were collected and ground to fine power. 30 mg leaf power was homogenized in 2 ml of 3% sulphosalicylic acid (SA), vortexed and then

centrifuged at 4000 rpm for 10 min. 500 µL of the supernatant was taken into a glass tube and 500 µL 3% SA was added, followed by adding 1 mL ninhydrin acid and 1 mL glacial acetic acid. The homogenate was heated at 100°C for 1 hour in water bath, and then quickly cooled in the ice bath. 2 ml toluene was then added to each tube and vibrated for a while. Tubes were kept at room temperature for at least 10 min to allow phase separation until the bottom layer became clear. The absorbance of upper layer with toluene was read at 520 nm. Proline content was determined by a standard curve from known concentrations of L-proline. The proline content in control samples was not detectable for the dilutions used in this method; hence, only proline content under drought and salinity stress are presented.

4.2.6 QTL analysis

A molecular map of this population has been published earlier (von Korff et al. 2008). The software package MapQTL 6.0 (Van Ooijen 2009) was used to detect QTLs which were first analysed by interval mapping (IM). The closest marker at each putative QTL identified using interval mapping was selected as a cofactor and the selected markers were used as genetic background controls in the approximate multiple QTL model (MQM). Logarithm of the odds (LOD) threshold values applied to declare the presence of a QTL were estimated by performing the genome wide permutation tests implemented in MapQTL version 6.0 using at least 1000 permutations of the original data set for each trait, resulting in a 95% LOD threshold around 3.0. To determine the effects of other traits on the QTLs for drought and salinity tolerance, QTL for both drought and salinity tolerance were re-analysed by using various agronomic traits (heading dates and awn length reported by Wang (Wang et al. 2010)) and physiological traits as covariates. Two LOD support intervals around each QTL were established, by taking the two positions, left and right of the peak, that had LOD values of two less than the maximum (Van Ooijen 2009), after performing restricted MQM mapping which does not use markers close to the QTL. The percentage of variance explained by each QTL (R^2) was obtained using restricted MQM mapping implemented with MapQTL6.0. Graphical representation of linkage groups and QTL was carried out using MapChart 2.2 (Voorrips 2002).

4.3 Results

4.3.1 Drought, salinity tolerance of the DH lines and proline contents under different stresses

DH lines from the cross between TX9425 and Franklin showed significant difference in drought or salinity tolerance and proline content ($P < 0.01$). Figure 4.2 shows the frequency distribution of drought tolerance (DT; based on leaf wilting), salinity tolerance (ST) based on plant survival scores, and proline content under drought (PC-D) and salinity (PC-S) stress for 72 lines. Continuous distributions were found for all the traits with wilting scores ranging from 4 – 9 for DT, 0 - 6 for ST, 1.2 -229.9 for PC-D and 7.1 - 49.6 for PC-S. Transgressive segregation was found for all three traits.

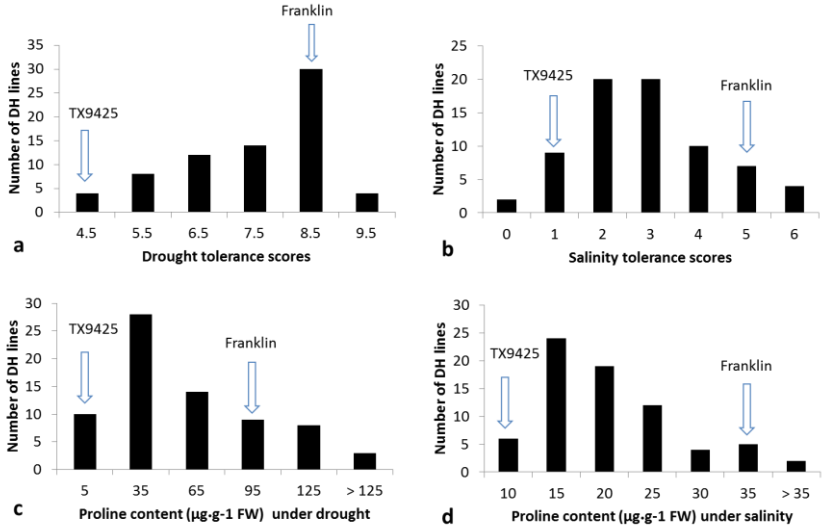

Figure 4.2 Frequency distribution for drought (a)/salinity (b) tolerance and proline content under drought(c)/salinity (d) stress of DH lines derived from the cross of TX9425/Franklin. (For both drought and salinity tolerance score, 0 = tolerant without obvious damage while 10 = sensitive and nearly dead)

4.3.2 QTL for different traits

QTL analysis indicated that both drought and salinity tolerance alleles were from tolerant parent TX9425. Two QTL for drought tolerance were identified on chromosomes 2H and 5H which were denoted as *QDT.TxFr.2H* and *QDT.TxFr.5H*, respectively (Figure 4.3, Table 4.1). bPb-7229 is the nearest marker for *QDT.TxFr.2H*, explaining 42.2% of phenotypic variation.

QDT.TxFr.5H explained 14.0% of phenotypic variation, with bPb-3700 being the closest marker. Relative water content showed a very close correlation ($r = 0.73$, $P < 0.01$) with drought tolerance (wilting scores) (Figure 4.4a). One QTL (*QRMO.TxFr.2H)* for RWC was identified on a similar position to *QDT.TxFr.2H* on 2H, and it explained 44.3 % of phenotypic variation. bPb-7229 is also the closest marker for this QTL. One QTL for proline content under drought conditions was found on 3H, explaining 32.0% of the phenotypic variation. This QTL was at different position to that for drought tolerance, indicating that drought tolerance and proline production under drought stress was controlled by different gene(s). This is further confirmed by correlation analysis that the changes of proline content under drought treatment showed no significant correlation with drought tolerance (Figure 4.4b).

Only one significant QTL *QST.TxFr.7H* controlling salinity tolerance (estimated by plant survival under salt stress) was found on 7H with a nearest marker bPb-6821. It explained 28.2% of phenotypic variation with a LOD value of 5.17 (Figure 4.3, Table 4.1). One QTL was identified for Na^+ content on 2H, determining 21.8% the phenotypic variation. This QTL is located at a similar position of that for drought tolerance with bPb-7229 being the closest marker for this QTL. Under salinity stress, some lines showed a significant increase in proline content. A QTL was identified for proline content on 3H, located on a similar position of the QTL for proline content under drought stress.

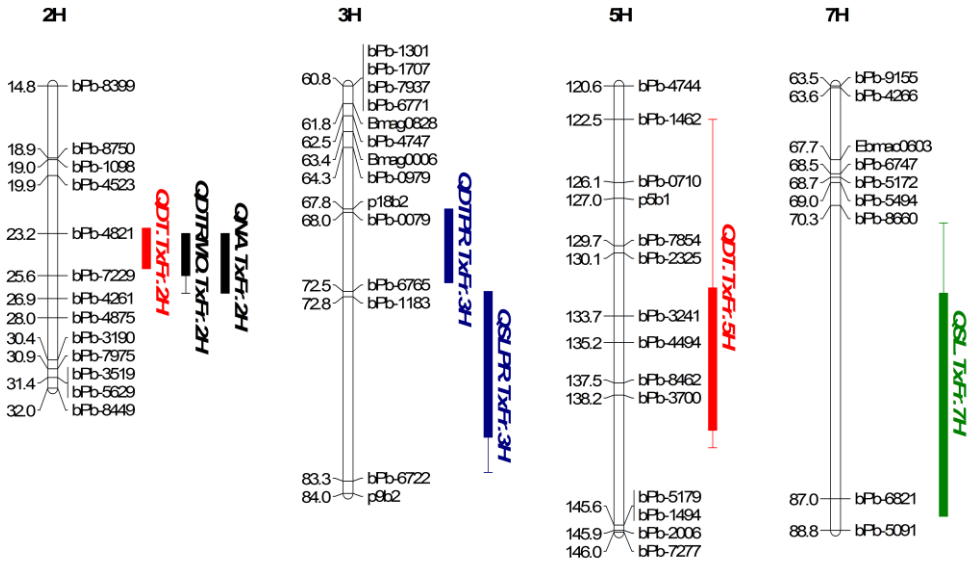

Figure 4.3 QTL associated with drought tolerance (in red), salinity tolerance (in green) and proline content under drought or salinity stress (in blue). For better clarity, only parts of chromosome regions were shown.

Table 4.1 QTLs for agronomic traits detected in the DH population of TX9425 × Franklin (average values)

Traits	QTL	Linkage group	Nearest Marker	Position(cM)	LOD	R^2 (%)
Drought tolerance	QDT.TxFr.2H	2H	bpb-4821	24. 2	8.56	42.2
	QDT.TxFr.5H	5H	bpb-3241	133.7	4.13	14
Salinity tolerance	QST.TxFr.7H	7H	bpb-6821	82.3	5.4	29.2
RWC	QRMO.TxFr.2H	2H	bpb-7229	25. 2	9.45	45.4
PC-D	QPC-D.TxFr.3H	3H	bpb-0079	70.0	6.65	34.7
PC-S	QPC-S.TxFr.3H	3H	bpb-6765	74.8	3.22	18.6
Na^+ content	QNA.TxFr.2H	2H	bpb-7229	25. 2	3.84	21.8

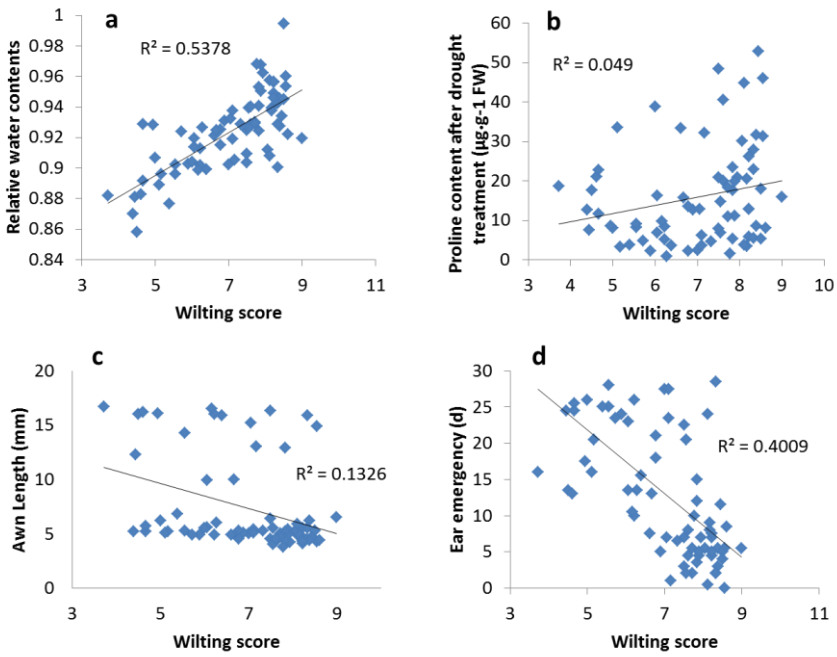

Figure 4.4 Correlation analysis. a: correlation analysis between RWC (relative water content) and drought tolerance (wilting score); b: correlation analysis between proline content (under drought stress) and drought tolerance; c: correlation analysis between AL (awn length) and drought tolerance; d: correlation analysis between EE (ear emergency) and drought tolerance.

Table 4.2 Correlation coefficients between different traits

	DT	RWC	PC-D	ST	PC-S	Na^+ content	EE
RWC	0.73						
PC-D	0.22	0.14					
ST	-0.47	-0.35	-0.12				
PC-S	-0.07	-0.10	0.16	0.37			
Na^+ content	-0.55	-0.37	-0.39	0.25	-0.10		
EE	-0.63	-0.51	-0.41	0.33	-0.10	0.65	
AL	-0.36	-0.43	-0.04	0.39	0.21	-0.04	-0.02

*Significance level: $r_{0.05}$ =0.23; $r_{0.01}$ =0.30

4.3.3 The effect of development genes on QTL for drought tolerance

PC-D, *RWC* and development genes (*uzu* gene and genes for ear emergency) showed significant correlation with drought tolerance (Table 4.2 and Figure 4.4). To further confirm the relationships between different traits, a QTL analysis was conducted by using different traits as covariates. Awn length (AL) was used to represent the existence of *uzu* gene (Chen et al. 2012a) and ear emergency (EE) data were from previous glasshouse trial (Wang et al. 2010) which had a similar condition to the experiments for both salinity and drought tolerance.

Table 4.3 lists QTL analysis for drought tolerance by using different traits as covariates. Of the two QTL for drought tolerance, *QDT.TxFr.5H* was less effected, which showed only slight reduction in R^2 when using RWC and EE as covariates. In contrast, *QDT.TxFr.2H* was significantly affected by genes controlling ear emergency. The QTL, which is located on a similar position to that for RWC and one of the QTL for ear emergency, became insignificant when using either EE or RWC as a covariate. A new QTL for drought tolerance was identified on 3H when using EE as a covariate. This QTL was dependent on the *uzu* gene as it disappeared when AL was also used as a covariate. As expected, proline content under drought treatment showed little effects on R^2 of both QTL for drought tolerance (Figure 4.5).

Table 4.3 QTL for drought tolerance when different physiological and developmental traits were used as covariates

QTL	Covariate	Linkage group	Nearest Marker	Position(cM)	LOD	R^2 (%)
QDT.TxFr.2H	Heading Date				ns	ns
QDT.TxFr.3H		3H	bpb-0079	67.3	4.5	10.9
QDT.TxFr.5H		5H	bpb-3241	133.7	3.51	8
QDT.TxFr.2H	Awn Length	2H	bpb-4821	24. 2	10.0	35.6
QDT.TxFr.5H		5H	bpb-3241	133.7	5.03	15.1
QDT.TxFr.2H	Awn Length + heading date				ns	ns
QDT.TxFr.5H		5H	bpb-3241	133.7	3.51	8.2
QDT.TxFr.2H	Proline	2H	bpb-4821	24. 2	9.82	38.0
QDT.TxFr.5H		5H	bpb-3241	133.7	4.88	15.9

*ns: not significant

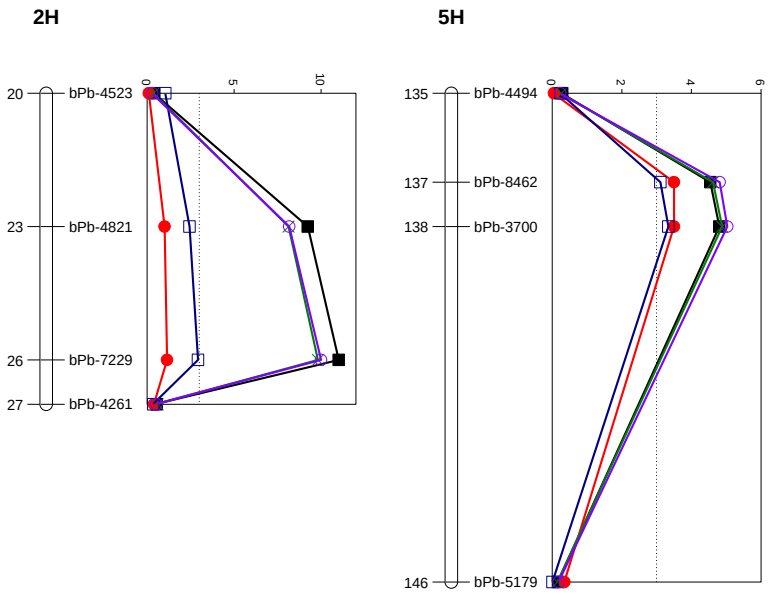

Figure 4.5 QTLs associated with drought tolerance (LOD values) on 2H and 5H. Black solid block: LOD value of original QTL; Purple hollow circle: LOD value of QTL when awn length was used as covariate; Green cross: LOD value of QTL when proline content was used as covariate; Red solid circle: LOD value of QTL when awn length and heading date were used as covariate; Blue hollow block: LOD value of QTL when heading date was used as covariate.

4.3.4 The effect of PC-S, RWC, Na^+ content and development genes on QTL for salinity tolerance

PC-S, *RWC* and development genes (*uzu* gene and genes for ear emergency) also showed significant correlation with salinity tolerance (Table 4.2). However, when a QTL analysis was conducted by using these traits as covariates, very minor effects were shown with the percentage of the phenotypic variation determined by the QTL (R^2) being reduced from 28% to around 20%, indicating that the tolerance gene is most likely independent of these traits. Na^+ content showed relatively low correlation (Table 4.2) with salinity tolerance, which is consistent with above results that the QTL for salinity tolerance and Na^+ contents under salt treatment were located at different chromosomes.

4.3.5 The effect of development genes on QTL for physiological traits

Developmental genes showed huge effects on the QTL for both proline and Na^+ contents. No significant QTL were detected for proline contents under both drought and salinity stress when using awn length as a covariate. The QTL on 2H for Na^+ contents under salinity stress became insignificant when using ear emergency as a covariate.

Even though that QTL for drought tolerance and salinity tolerance were located on different chromosomes, the R^2 of the QTL for salinity tolerance was reduced from 28% to around 16% when using drought tolerance scores as a covariate. Likewise, the R^2 of the QTL for drought tolerance on 2H was reduced from 44% to around 32% when using salinity tolerance scores as a covariate.

4.4 Discussion

4.4.1 QTL on 5H for drought tolerance is less affected by plant height and maturity

Drought is one of the major abiotic yield-limiting factors in crops which have been affected by early season water deficit worldwide. Therefore, understanding the genetic background and enhancing drought tolerance is crucial for both breeding and basic research. Owing to the complexity of drought, strong QTL-environment interaction, possible epistatic effects and small explanation of drought tolerance loci, the knowledge on drought tolerance is still incomplete (Li et al. 2001; Teulat et al. 2001a). In barley, QTL analysis for numerous traits has been performed under drought stress or Mediterranean rainfed conditions including leaf wilting (Sayed et al. 2012b), proline content (Sayed et al. 2012b), chlorophyll content (Guo et al. 2008), relative water content (Diab et al. 2004; Teulat et al. 2001a; Teulat et al. 2003), osmotic adjustment (Teulat et al. 2001a), carbon isotope discrimination (Chen et al. 2012b), water-soluble carbohydrate concentration (WSC) (Diab et al. 2004; Teulat et al. 2001a), flowering time or heading date (Szira et al. 2011; Teulat et al. 2001b), plant height (Teulat et al. 2001b; von Korff et al. 2008), grain yield and seed quality parameters (Kalladan et al. 2013; Szira et al. 2011). However, most of them are not dealing with the drought tolerance which is the changes of the traits under drought conditions compared to controls. In this study, we used leaf wilting as a major index for drought tolerance (no wilting was shown in controls) and identified two QTLs controlling drought tolerance on 2H (*QDT.TxFr.2H*) and 5H (*QDT.TxFr.5H*). The co-localization of *QDT.TxFr.2H* and another QTL for relative moisture

(*QRMO.TxFr.2H*) suggested a common genetic control between them and the possibility for RMO under drought stress used as selection criteria for drought tolerance. However, *QDT.TxFr.2H* and *QRMO.TxFr.2H* were located in the similar position as a QTL conferring heading date on 2H which was identified by Wang (Wang et al. 2010). When QTLs for heading date and awn length (Wang et al. 2010) was added to the analysis as covariates, *QDT.TxFr.2H* could not be detected (Table 4.3), indicating that these two traits were dependent on the development genes. Even though no association was found between drought tolerance and heading date in one of the reports (Samarah et al. 2009), drought escape via a short life cycle, together with drought avoidance, drought tolerance and drought recovery are crucial mechanisms of drought resistance. Under drought stress, early flowering is a beneficial trait for plants to escape from stress at the expense of reduced yield potential (Ludlow and Muchow 1990). In contrast to *QDT.TxFr.2H, QDT.TxFr.5H* was less affected by different development genes (Table 4.2). Thus *QDT.TxFr.5H* could be a candidate locus for further drought tolerance study. The development of near isogenic lines based on this locus should be the best approach to avoid the interference of other development genes and to fine map this gene.

4.4.2 Salinity tolerance identified from this population was not linked with Na^+ absorption

Salinity tolerance is controlled by multi-gene traits where genes are expressed at various plant developmental stages. A large number of agronomic and physiological indices were used to quantify plant salinity stress tolerance including seed germination (Witzel et al. 2010), plant survival (Xu et al. 2012; Zhou et al. 2012), Na^+ exclusion (Shavrukov et al. 2010), tissue ion content (Xue et al. 2009), yield and agronomic traits (Ellis et al. 2002; Xue et al. 2009), chlorophyll content and water soluble carbohydrate (Siahsar and Narouei 2010). In the current experiment, plant survival under saline conditions was scored at seedling stage and one major QTL for salinity tolerance (*QST.TxFr.7H*) was identified on chromosome 7H (Figure 4.3). This QTL was at a similar position to the one (*QST.YyFr.7H*) recently identified by Zhou et al. (2012) and another trait *HvNax3* on 7H controlling shoot Na^+ exclusion identified by Shavrukov et al. (2010). However, in this population, leaf Na^+ content showed no correlation with salinity tolerance. The difference between this work and Shavrukov et al. (2010) could be due to the different genotype involved or different experimental set up such as growth condition and salt concentration applied. The most likely explanation for this is

that in above studies plants were treated with much lower levels of NaCl. Under these conditions, plants were able to osmotically adjust to relatively mild hyperosmotic stress by *de novo* synthesis of compatible solutes and, hence, did not relied on the use of Na^+. In our work, 320 mM NaCl was used to screen plants. Osmotic adjusting to this stress by *de novo* synthesis of compatible solutes would come at a huge metabolic cost (Shabala and Shabala 2011), and Na^+ uptake into leaf was energetically more favourable option (on a provision it is effectively sequestered in the vacuole). As leaf Na^+ analysis for QTL mapping was done at the whole-tissue level and did not differentiate between the Na^+ distribution between the cytosol and the vacuole, the lack of correlation between Na^+ content and salt tolerance is hardly surprising.

4.4.3 The changes of proline content under drought and salinity stresses are not necessarily linked to drought and salinity tolerance

Under control conditions, proline is needed to participate in normal metabolisms and regulate plant developmental processes(Polidoros and Scandalios 1999). Various abiotic stresses can induce proline biosynthesis (Szabados and Savoure 2010) to balance osmotic pressure in cells, maintain redox balance and activate signalling networks for stress adaption (Polidoros and Scandalios 1999). In the current study, proline level increased in plants exposed to both drought and salinity stress. QTLs for proline contents under drought (*QPC-D.TxFr.3H*) and salinity stress (*QPC-S.TxFr.3H*) were identified to be at the similar positions. However, they were at different positions with QTLs for either drought (*QDT.TxFr.2H, QDT.TxFr.5H*) or salinity stress (*QST.TxFr.7H*) tolerance (Figure 4.3, Table 4.1). QTL analysis for drought and salinity tolerance using proline content as a covariate further confirmed that there was no correlation between proline accumulation and tolerance to either stress. The results suggested that proline biosynthesis under drought or salinity stresses is not necessarily linked to drought or salinity tolerance. As commented above, high metabolic cost of proline biosynthesis may be the reason.

Interestingly, QTL conferring proline content under abiotic stress were at the similar position to the QTL for awn length on chromosome 3H with bpb-0079 as closest marker (Chen et al. 2012a; Wang et al. 2010). As shown in Table 4.2, *QPC-D.TxFr.3H* was disappeared after adding awn length as covariate for QTL analysis, indicating that proline biosynthesis may have some cross-talks with plant development. Increasing data from over-expressions or knock-out mutants of proline synthesis genes indicate that proline participates in embryo and

plant development (Szekely et al. 2008), influences leaf or inflorescences morphology (Nanjo et al. 1999) and affects blossoms time (Mattioli et al. 2008).

In conclusion, QTL mapping approach was used in this study to determine the linkages between stress tolerance and different physiological and developmental traits. A QTL on 5H for drought tolerance was less affected by other developmental traits and this locus can be effectively used in breeding programs.

Chapter 5 QTLs for stomatal and photosynthetic traits related to salinity tolerance in barley

5.1 Introduction

Soil salinity results from natural causes such as from soluble salts from rocks and oceanic salts carried in wind and rain as well as from increasing salinization of agricultural land due to irrigation and deforestation (Munns and Tester 2008; Rengasamy 2002); this salinization of agricultural land is increasing due to global climate change. Salinity is causing major, global, food security issues due to the large arable area that is now saline and not suitable for cropping; therefore, breeding salt tolerant crops has become an important priority. Genetic manipulation could produce transgenic plants containing novel genes or different expression levels of existing genes that will improve plant salt tolerance (Cuin and Shabala 2007b). However, salinity tolerance is controlled by multi-gene traits where genes are expressed at a number of plant developmental stages in a highly tissue-specific manner. Genetic engineering of single genes has proved problematic for improving salt tolerance in crops (Schroeder et al. 2013), and it is unlikely to be accepted by general public. However, molecular breeding could be used for breeding salt tolerant crops by exploiting existing genetic variation through direct selection or marker assisted selection in conjunction with the use of quantitative trait loci (QTLs) for gene pyramiding.

Stomata are formed by two highly specialised guard cells, and some are surrounded by subsidiary cells in certain plant species like barley (Bergmann and Sack 2007). Stomata control the exchange of water vapour and CO_2 between the leaf interior and the atmosphere, and serve as major gateways for CO_2 influx into plants as well as transpirational water loss from plants (Kim et al. 2010; Lawson and Blatt 2014; Laza et al. 2010). The transpirational water loss through stomatal pores contributes to 70% of total agricultural water use (Hetherington and Woodward 2003). The stomatal aperture is influenced by the plant and its environment (Ainsworth and Rogers 2007). Under saline conditions, plant cells lose water and reduce cell elongation for short-term osmotic adjustment and later build up cellular NaCl over a longer period (Munns and Tester 2008; Zhu 2002). This accumulation of NaCl in plant cells, including stomatal guard cells, affects their function. Stomatal closure is one of the

most immediate responses to salinity (Fricke et al. 2004; Fricke et al. 2006; Munns and Tester 2008), and this response is believed to be crucial for minimising plant water losses under hyperosmotic conditions in rhizosphere (El-Wahab et al. 2011; Reef and Lovelock 2014; Shabala and Pottosin 2014). Reducing the stomata density is another way of optimising the balance between leaf water loss and CO_2 assimilation, and it was shown that halophytes, naturally salt-tolerant species, are capable of reducing stomatal density when grown under hypersaline conditions (Shabala et al. 2012; Shabala et al. 2013). The same effect was observed in most tolerant barley varieties (Zhu et al. 2015). However, this strategy has a caveat, as the closure of stomata will reduce photosynthesis thereby reducing plant biomass and crop yield (Centritto et al. 2003).

Stomatal and photosynthetic parameters, such as stomatal size and frequency, stomatal conductance, photosynthesis, transpiration rate, and water use efficiency, affect the grain yields of crops under stressed and non-stressed conditions (Aminian et al. 2011; Khazaei et al. 2010). Genotypic variation in stomatal traits has been reported, but little is known about the genetic mechanisms behind these traits. A negative association between water loss and stomatal size was found in durum wheat (Venora and Calcagno 1991), and Wang and Clarke (Wang and Clarke 1993) reported a positive correlation between stomatal frequency and the rate of water loss in bread wheat. Stomatal paremeters (e.g., stomatal aperture, guard cell volume, aperture width and aperture width/length) were significantly different between salt-tolerant and salt-sensitive genotypes. Significant correlations have been found between stomatal traits, expression of slow anion channel genes and grain yield in salt-tolerant barley (Liu et al. 2014). Expression of slow anion channel gene may contribute to transportation anion such as Cl^- which is a major component of salt in soil, leading to the increase og grain yield. Correlation between stomatal traits and grain yiled suggests that stomatal traits may contribute to salinity tolerance in barley, but further study using genetic populations is required.

Many studies on the salinity tolerance of plants focus on ionic relations, but there has been little research to determine the potential role of stomatal function in salinity tolerance. QTLs for gas exchange or stomatal parameters under greenhouse conditions or different stresses have been identified in *Arabidopsis* (Juenger et al. 2005), rice (Laza et al. 2010; Price et al. 1997; Teng et al. 2004), sunflower (Herve et al. 2001), and faba bean (Khazaei et al. 2014). In barley, QTLs associated with net photosynthetic rate have been detected under drought stress (Wojcik-Jagla et al. 2013). In addition, Liu et al. (Liu et al. 2015) identified QTLs for

net photosynthetic rate and stomatal conductance in barley grown without stress. Also, three QTLs for stomatal density have been identified on chromosomes 1, 3 and 7 using 100 lines of an F_2 population from the cross between two *Hordeum chilense* accessions. The two QTLs on chromosome 3 overlapped with a QTL for avoidance of leaf rust, previously identified at the same genomic region. However, to the best of our knowledge, QTLs for stomatal traits, especially stomatal aperture and guard cell and subsidiary cell geometry under salinity stress, have not been reported in plants.

In our recent work, we have explored stomatal and photosynthetic traits as potential selection criteria for plant salt tolerance (Liu et al. 2014). Here, we measured those stomatal and photosynthetic traits in a double haploid (DH) population of barley to identify significant QTLs. We hypothesised that stomatal traits are controlled by multiple genes and would result in multiple QTLs for salt tolerance in barley. Thus, the objectives of this study were to: (1) identify QTLs for stomatal and photosynthetic traits associated with salinity tolerance in barley, and (2) investigate the relationships between salinity tolerance and stomatal regulation through QTL mapping.

5.2 Materials and methods

5.2.1 Plant materials and growth conditions

A barley DH population consisting of 108 lines from a cross between CM72 (salt-tolerant) and Gairdner (salt-sensitive) were used. Seeds of the two parental cultivars were conducted at the Hawkesbury Campus of Western Sydney University, Australia. Seeds (5 per pot) were germinated and grown in 4 L pots containing potting mix (Debco Pty Ltd, Victoria, Australia) augmented with 5 g Osmocoat® slow release fertiliser. Two parallel trials were conducted in two glasshouse rooms with grow lamps (600 W) at a temperature of 25±1 °C, 65% relative humidity (RH) and a photoperiod of 12/12 h light/dark. Prior to treatment with NaCl, all plants were watered twice weekly and fertilised with Hoagland's solution. The plants were subjected to NaCl treatment beginning at Week 5 after sowing commencing with the addition of NaCl over 4 consecutive days to final concentration 200 mM NaCl in an attempt to avoid osmotic shock. All leached salt was collected in a saucer under the pot and re-applied to ensure stability of concentrations across all treatment pots. The soil electrical conductivity (EC) was measured regularly. Four weeks after salt treatment, gas exchange and stomatal assay were conducted. Grain yield, biomass and row type were determined at Week 20.

Normal pest control, fertiliser and Hoagland's solution application were employed. In addition, three glasshouse trials evaluating the salinity tolerance of the CM72/Gairdner DH population were conducted at Launceston, Tasmania, Australia. Plant growth conditions and salt treatment were similar to those previously described (Zhou et al. 2012).

5.2.2 Gas exchange measurements

Physiological measurements were made according to O'Carrigan et al. (O'Carrigan et al. 2014) to determine net photosynthetic rate, intercellular CO_2 concentration, stomatal conductance, transpiration rate, leaf vapour pressure deficit, and leaf temperature. Measurements were taken with four LI-6400XT infrared gas analysers (Li-Cor Inc., Lincoln, NE, USA), using the third fully expanded leaves of seedlings 4 weeks after the salt treatment ended. The measuring chambers had an air flow rate of 500 mol s^{-1}, a saturating photosynthetically active radiation (PAR) of 1500 μmol m^{-2} s^{-1}, a CO_2 concentration of 400 μmol mol^{-1} and a relative humidity of 65%. Gas exchange measurements were taken at the same time (approximately 10 a.m. to 4 p.m.) as those for stomatal assays.

5.2.3 Measurement of stomatal parameters

Twelve stomatal traits were analysed as described by Liu et al. (Liu et al. 2014), Mak et al. (Mak et al. 2014) and O'Carrigan et al. (O'Carrigan et al. 2014). The parameters were aperture length (AL), aperture width (AW), aperture width/length (AWL), stomatal pore area (SA), guard cell length (GCL), guard cell width (GCW), guard cell volume (GCV), subsidiary cell length (SCL), subsidiary cell width (SCW), subsidiary cell volume (SCV), stomatal density (SD), and stomatal index (SI). For these measurements, the third fully expanded leaves were collected from the glasshouse and placed on tissue paper soaked in a stabilising solution (50 mM KCl, 5 mM Na^+-MES, pH 6.1) in Petri dishes. Abaxial epidermal strips were then peeled and mounted on slides using a measuring solution (10 mM KCl, 5 mM Ca^{2+}-MES, pH 6.1). Quick peeling and mounting was important to ensure stomatal images were true representations of the stomata found naturally on the whole plant in the glasshouse. Stomatal imaging was conducted using a CCD camera (NIS-F1 Nikon, Tokyo, Japan) attached to a microscope (Leica Microsystems AG, Solms, Germany). All images were analysed using a Nikon NIS Element imaging software (Nikon, Tokyo, Japan) and measured with Image J software (NIH, USA).

5.2.4 Salinity tolerance score

Salt tolerance was assessed at the seedling stage by combining scores for leaf chlorosis and plant survival (0 = no damage and 10 = all dead) when the most susceptible lines showed severe symptoms (Xu et al. 2012). In this study, salt tolerance evaluation was based on the average value from results obtained in 2010, 2014 and 2015.

5.2.5 QTLs and statistical analysis

The data regarding photosynthetic and stomatal traits as well as biomass and grain yield under control and saline condition were used for QTL analysis. The ratios of these traits in saline to control conditions were also tested for QTL identification. A genetic linkage map for this population was constructed using Diversity Array Technology (DArT) and Simple Sequence Repeat (SSR) markers. The software package, MapQTL 6.0 (Van Ooijen 2009), was used to detect QTL. QTLs were first analysed by interval mapping (IM). Following this, the closest marker at each putative QTL identified with interval mapping was selected as a cofactor, and the selected markers were used as genetic background controls in the approximate multiple QTL model (MQM). A logarithm of the odds (LOD) threshold value of 3.0 which is estimated by performing the genome wide permutation tests using at least 1000 permutations of the original data set for each trait was applied to declare the presence of a QTL at the 95% significance level. To determine the effects of other traits on QTLs for salinity tolerance, the QTLs for salinity tolerance were re-analysed using other traits as covariates. Two LOD support intervals around each QTLs were established, by taking the two positions, left and right of the peak, that had LOD values of two less than the maximum (Van Ooijen 2009), after performing restricted MQM mapping which does not use markers close to the QTL. The percentage of variance explained by each QTL (R^2) was obtained using restricted MQM mapping implemented with MapQTL 6.0. Graphical representation of linkage groups and QTLs was carried out using MapChart 2.2 (Voorrips 2002). Frequency distribution analysis was performed using SigmaPlot 12 (Systat Software Inc., San Jose, CA, USA).

5.2.6 Genomic analysis of potential genes for salinity tolerance

The sequence marker, Bmac0209, associated with the QTL for salinity tolerance on 3H was used to identify candidate genes for salinity tolerance. The genome sequence of this region

was retrieved by BLAST search on the website (http://webblast.ipk-gatersleben.de/barley/). A Morex contig, 84335, was found to be homologous with Bmac0209. The physical map position of this contig was located at 51.77 cM on 3H. Barley genomic data and gene annotations were downloaded from ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/ (Consortium 2012) and ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/popseq_IPK/ (Mascher et al. 2013). Annotated genes between 46.74 and 56.72 cM deemed to be potential genes for salinity tolerance (Appendix 5.1).

5.3 Results

5.3.1 The DH and parental lines show a large diversity in salinity tolerance

The 108 DH lines showed significant differences in salinity tolerance, with CM72 being scored as 1 and Gairdner at 5. Grain yield, biomass, leaf temperature, transpiration rate, stomatal area and other parameters of DH lines under control or saline conditions displayed continuous frequency distributions (Figures 5.1–3). Salinity stress caused a significant shift in the distribution of photosynthetic and stomatal traits and in grain yield (Figures 5.1, 2, 3C and 3D). Stomatal conductance, transpiration rate, and intracellular CO_2 concentration showed a distribution skewed to lower values under salt stress whereas leaf vapour pressure deficit and leaf temperature displayed a distribution skewed to higher ranges (Figure 5.1). For stomatal traits, stomatal pore area, aperture width/length and subsidiary cell length had distributions skewed to lower values under salt stress; in contrast, subsidiary cell width and subsidiary cell volumes showed a distribution skewed to higher ranges (Figure 5.2). Grain yield was skewed to lower values under salinity treatment. Of the two parents, CM72 showed better performance than Gairdner for all traits under saline stress (Figures 5.1–3). All traits showed transgressive segregation at both sides, indicating that both parents transmitted favourable alleles for each trait (Figures 5.1–3). This enabled the QTL mapping to identify a total of 11 QTLs with LOD values of > 3.0 (Figure 5.4, Table 5.1).

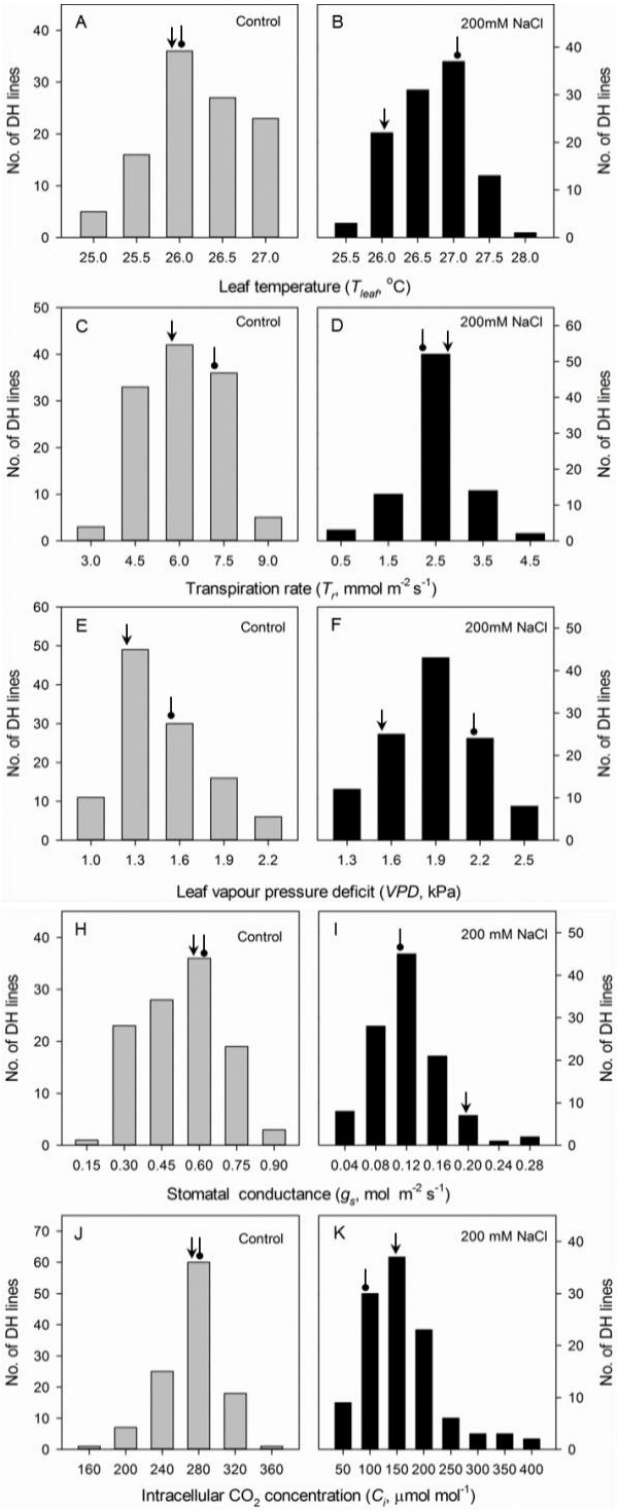

Figure 5.1 Frequency distribution of gas exchange traits in control and salt treatment. Shown are leaf temperature (A, B), transpiration rate (C, D), leaf vapour pressure deficit (E, F), stomatal conductance (H, I) and intracellular CO_2 concentration (J, K) of DH lines derived from the cross of CM72 and Gairdner. Arrow represents CM72 and oval arrow are Gairdner. Data are averages of four replicates.

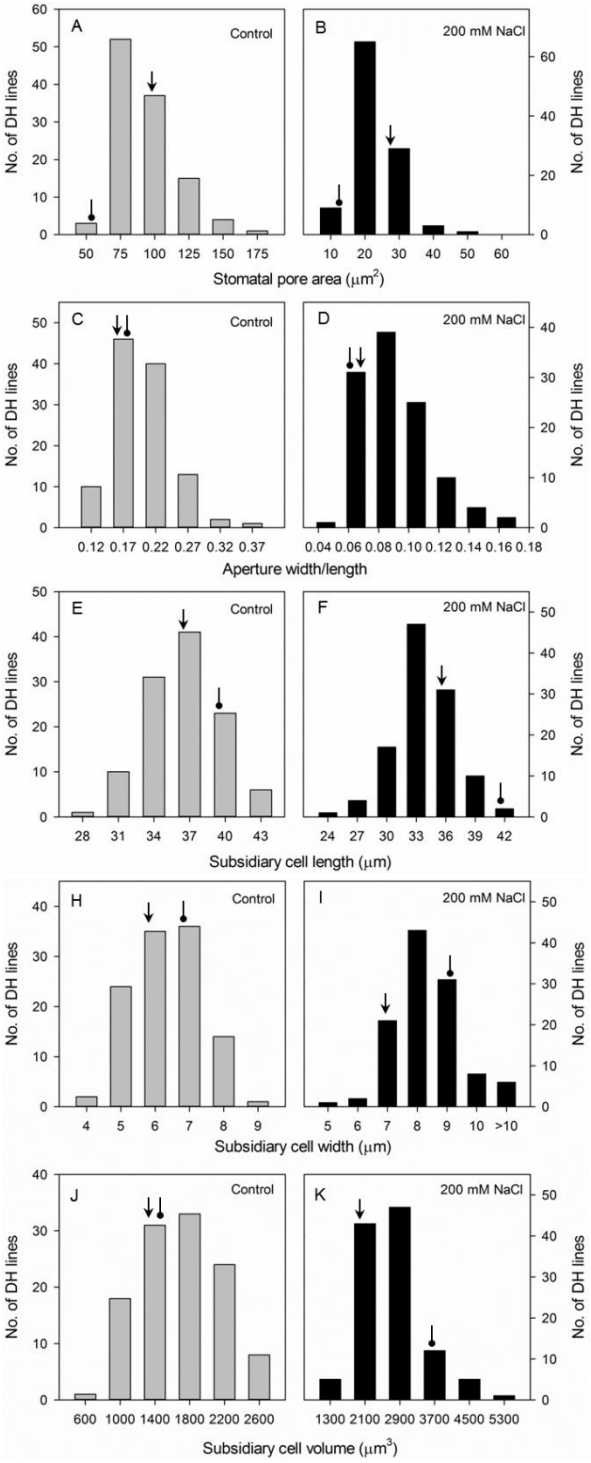

Figure 5.2 Frequency distribution of stomatal traits in control and salt treatment. Shown are stomatal pore area (A, B), aperture width/length (C, D), subsidiary cell length (E, F), subsidiary cell width (H, I) and subsidiary cell volume (J, K) of DH lines derived from the cross of CM72 and Gairdner, under control and salinity treatment conditions. Arrow represents CM72 and oval arrow represents Gairdner. Data are averages of 16-73 cells from 4 replicates.

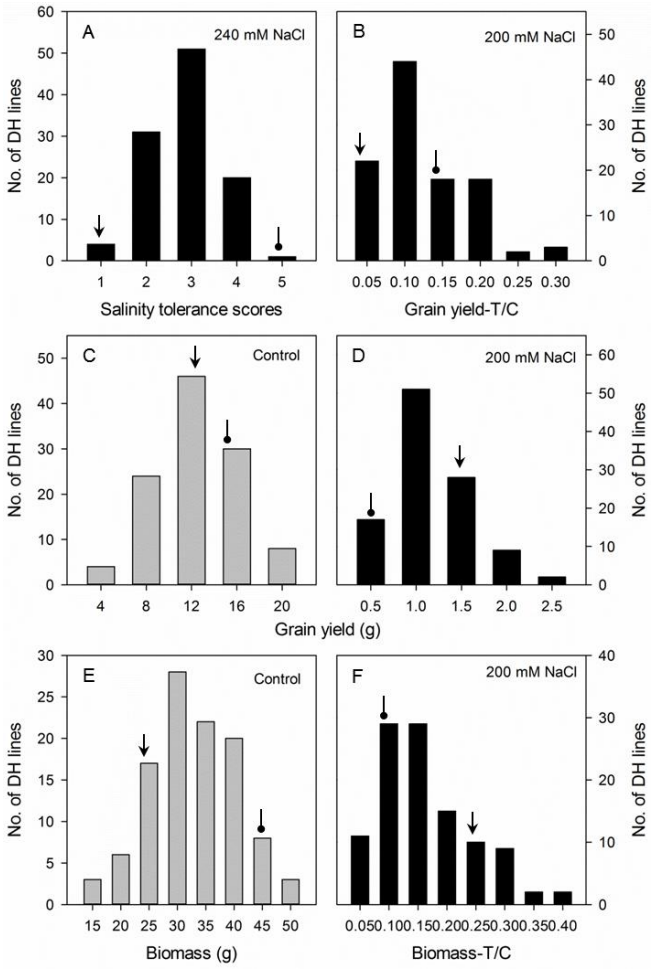

Figure 5.3 Frequency distribution for salinity tolerance score (A), relative grain yield-T/C (B), grain yield (C, D), biomass (E) and relative biomass-T/C (F) of DH lines derived from the cross of CM72 and Gairdner under control and salt treatment. T/C: the ratio of traits under salt treatment (T) and control (C). Arrow represents CM72 and oval arrow represents Gairdner. Data are averages of four replicates. Salinity tolerance score are averaged over three years with three replicates each year.

5.3.2 Significant QTLs for gas exchange and stomatal traits under control and saline conditions

One QTL for transpiration rate under control conditions (*QTR-C.CmGa.1H*) was detected on chromosome 1H close to bpb-1381 and explained 14% of the phenotypic variation. Two QTLs associated with leaf temperature were identified at same position on chromosome 2H, *QLT-C.CmGa.2H* under control conditions and *QLT-T.CmGa.2H* in the salinity treatment, explaining 16.5% and 11.2% of the phenotypic variation, respectively (Table 5.1, Figure 5.4). Only one significant QTL, *QSA-T.CmGa.1H* associated with stomatal pore area under salinity

treatment, was found on the long arm of chromosome 1H; this had a LOD value of 3.12. This QTL, with the closest marker bPb-9081, accounted for 12.5% of the phenotypic variation (Figure 5.4, Table 5.1).

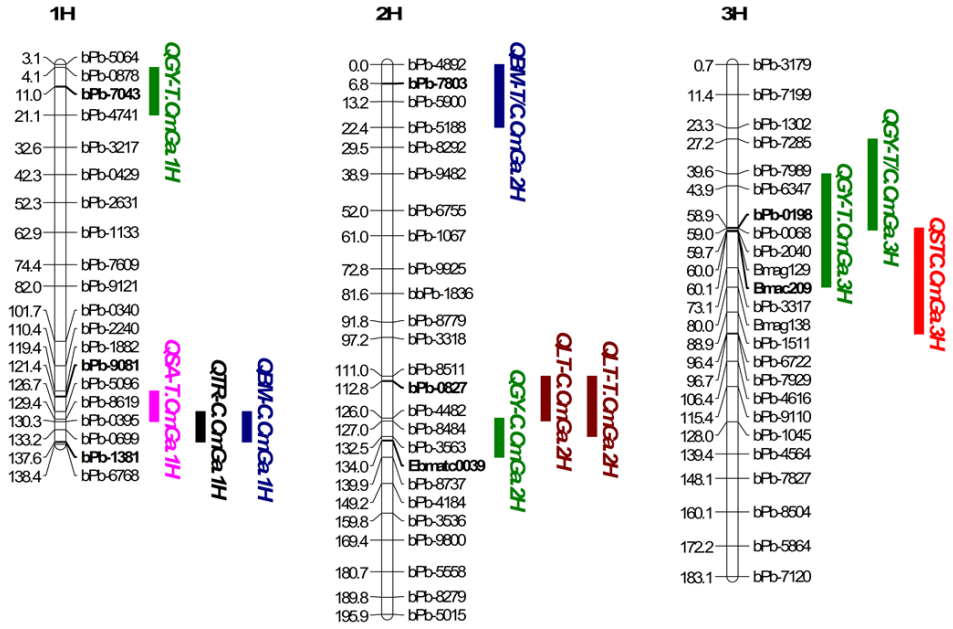

Figure 5.4 QTLs associated with salinity tolerance (in red), grain yield (in green), biomass (in blue), stomatal area (in pink), transpiration rate (in black) and temperature of leaves (in brown). For clarity, only part of the chromosome regions which cover 2-LOD interval of all the QTLs are shown. C: Control; T: salt treatment.

5.3.3 Significant QTLs for grain yield, biomass and salinity tolerance under control and saline conditions

Four QTLs for grain yield were found on chromosomes 1H, 2H and 3H: *QGY-T.CmGa.1H* and *QGY-T.CmGa.3H* under salinity stress; *QGY-C.CmGa.2H* under control conditions; and *QGY-T/C.CmGa.3H* based on the ratio of salinity stress relative to control. *QGY-C.CmGa.2H* (with Ebmatc0039 as the nearest marker) explained 15.0% of the phenotypic variation and *QGY-T.CmGa.1H* (with bPb-7043 as the closest marker) accounted for 13.9% of the phenotypic variation (Figure 5.4; Table 5.1). Moreover, two QTLs for biomass were identified on chromosomes 1H and 2H, respectively. The QTL, *QBM-C.CmGa.1H*, found under control conditions and flanked by bPb-1381, explained 15% of the phenotypic variation. In contrast, *QBM-T.CmGa.2H*, found under salinity stress with the nearest marker

Table 5.1 QTL and tentative QTL identified for different traits under saline and control conditions in a double haploid population derived from a cross between CM72 and Gairdner.

Trait	QTL	Linkage group	Position	LOD	R^2 (%)	Nearest marker
GY-T	*QGY-T.CmGa.1H*	1H	11	3.95	12.5	bPb-7043
SA-T	*QSA-T.CmGa.1H*	1H	121.4	3.12	12.5	bPb-9081
TR-C	*QTR-C.CmGa.1H*	1H	137.6	3.52	14	bPb-1381
BM-C	*QBM-C.CmGa.1H*	1H	137.6	3.79	15	bPb-1381
GS-C		1H	137.6	2.76	11.2	bPb-1381
CI-C		1H	137.6	2.75	11.2	bPb-1381
BM-T/C	*QBM-T.CmGa.2H*	2H	6.8	3.49	14	bPb-7803
TR-T/C		2H	6.8	2.54	10.4	bPb-7803
GS-T		2H	10.3	2.54	10.3	bPb-1949
LT-C	*QLT-C.CmGa.2H*	2H	112.8	4.19	16.5	bPb-0827
LT-T	*QLT-T.CmGa.2H*	2H	112.8	3.09	11.2	bPb-0827
GY-C	*QGY-C.CmGa.2H*	2H	134	3.77	15	Ebmatc0039
VPD-T/C		2H	153.6	2.63	10.7	bPb-2701
GY-T	*QGY-T.CmGa.3H*	3H	58.9	4.35	13.9	bPb-0198
GY-T/C	*QGY-T/C.CmGa.3H*	3H	58.9	4.42	17.3	bPb-0198
STC	*QSTC.CmGa.3H*	3H	60.1	4.29	16.8	Bmac209
AWL-T		3H	136.9	2.78	11.3	bPb-3634
SCL-T		4H	122.5	2.98	12	bPb-6153
LT-T		5H	53.3	2.69	9.7	bPb-2762
SCW-C		6H	93.5	2.81	11.4	EBmac602
SCV-C		6H	93.5	2.58	10.5	EBmac602
LT-C		7H	63.9	2.81	9.5	bPb-1209

*Abbreviations: **-C**: traits under control conditions; **-T**: traits under salt treatment; **-T/C**: the ratio of traits under salt treatment to traits under control conditions; **GY**: grain yield; **BM**: biomass; **STC**: salinity tolerance score; gas exchange traits: **TR**: transpiration rate; L**T**: leaf temperature; **GS**: stomatal conductance; **CI**: intercellular CO_2 concentration; **VPD**: leaf vapour pressure deficit; **SA**: stomatal pore area; **AWL**: aperture width/length; **SCL**: subsidiary cell length; **SCW**: subsidiary cell width; **SCV**: subsidiary cell volume. Tentative QTL: 2.5 < LOD < 3.0. QTL: LOD > 3.0

bPb-7803, is located near the telomere of the short arm of chromosome 2H and explained 14% of the phenotypic variation (Figure 5.4; Table 5.1). Furthermore, *QST.CmGa.3H* (60.1 cM) associated with salinity tolerance, was identified on chromosome 3H with Bmac209 being the nearest marker. This QTL explained 16.8% of phenotypic variation and had a LOD value of 4.29 (Figure 5.4, Table 5.1) with CM72 contributing to the tolerance.

5.3.4 Tentative QTL for gas exchange traits under control and saline conditions

Apart from the significant QTLs, 11 tentative QTLs ($2.5 < LOD < 3.0$) were also identified in this study (Figure 5.6, Table 5.1). A QTL (GS-C) for stomatal conductance and one for intercellular CO_2 concentration (CI-C) under control conditions were located on chromosome 1H near marker bPb-1381 (Figure 5.6, Table 5.1). There are three tentative QTLs on chromosome 2H including ones for stomatal conductance under saline conditions (GS-T), transpiration rate (TR-T/C) and vapour pressure deficit (VPD-T/C) found under salinity stress relative to control (Figure 5.6, Table 5.1). Also, a QTL for leaf temperature under salt stress (LT-T) and one (LT-C) under control conditions were located to chromosomes 5H and 7H, respectively (Figure 5.6, Table 5.1). In addition, four tentative QTL were identified for stomatal traits. One QTL for aperture width/length under salt stress (AWL-T) was found on chromosome 3H (Figure 5.6, Table 5.1). Another QTL for subsidiary cell length under stress (SCL-T) was identified on chromosome 4H, while QTL for subsidiary cell width (SCW-C) and subsidiary cell volume (SCV-C) under control conditions were both found on chromosome 6H (Figure 5.6, Table 5.1).

5.3.5 Co-localisation of phenotypic traits

There are five clusters of QTLs for different traits (Figure 5.4 and 5.6). On chromosome 1H, QTLs for transpiration rate (TR-C), biomass (BM-C), stomatal conductance (GS-C) and intercellular CO_2 concentration (CI-C) under control conditions were located at the same position (137.6 cM) and shared a common nearest marker, bpb-1381 (Table 5.1). On chromosome 2H, two QTLs for biomass and transpiration rate relative to control (Bm-T/C, TR-T/C) were both at 6.8 cM with bpb-7803 being the closest marker, while another QTL for stomatal conductance under stress (GS-T) was located at 10.3 cM close to *QBM-T/C.CmGa.2H* and *QTR-T/C.CmGa.2H* (Table 5.1). Moreover, QTLs for leaf temperature under control (LT-C) and saline conditions (LT-T) were at same position (112.8 cM) on chromosome 2H with bpb-0827 as the closest marker (Table 5.1). In addition, QTLs for grain

yield (GY-T, GY-T/C) on chromosome 3H (58.9 cM) were located close to a QTL for salinity tolerance (60.1 cM) (Figure 5.5, Table 5.1). Furthermore, QTLs for subsidiary cell width (SCW-C) and subsidiary cell volume (SCV-C) under control conditions were both at 93.5 cM on chromosome 6H with EBmac602 as the nearest marker (Table 5.1).

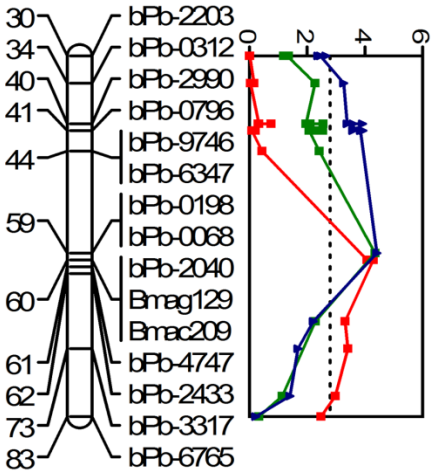

Figure 5.5 QTL associated with salinity tolerance (red solid line), grain yield-T (green solid line) and grain yield-T/C (blue solid line) located at similar position on 3H. The dotted line around LOD 3.0 is a line of significance. T: salt treatment; T/C: the ratio of traits under salt treatment (T) and control (C).

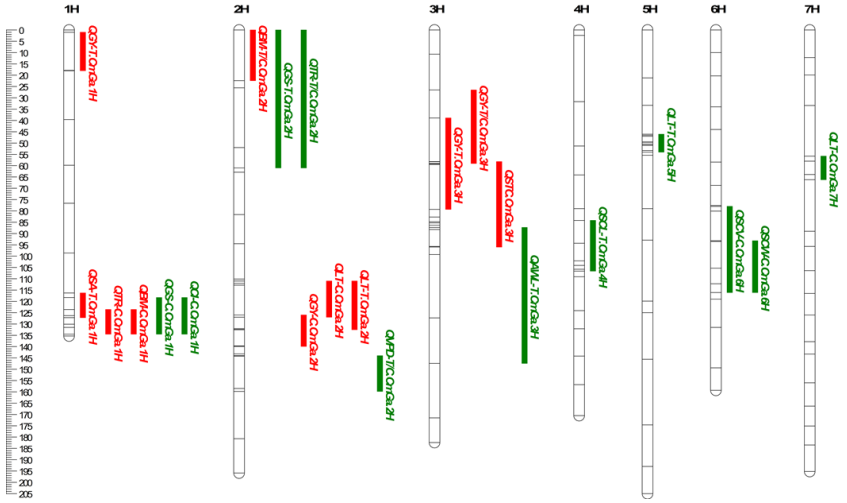

Figure 5.6 QTL associated with different traits of the DH lines derived from the cross of CM72 and Gairdner under control and salinity conditions. QTLs with significance (LOD>3.0) were shown in red colour, while QTLs in green colour are not significant but exhibit tendency (LOD>2.5). C: control; T: salt treatment; T/C: the ratio of traits under salt treatment (T) and control (C).

5.3.6 Identification of candidate genes for salinity tolerance on 3H

QTLs for grain yield relative to the control were located close to the QTL for salinity tolerance on chromosome 3H (Figures 5.4 and 5.5, Table 5.1). This led to further investigation of the possible genes controlling salinity tolerance in barley. Annotated genes close to SSR marker Bmac0209 were examined from the published barley genome sequence. There is a wide variety of potential salt tolerance related genes within this region of interest (Appendix 5.1). They are genes related to: reactive oxygen species (ROS) including a peroxidase (AK249079.1 at 51.63 cM) and a respiratory burst oxidase-like protein (MLOC_81745.1 at 50.67 cM); ion transport including a potassium channel (MLOC_74879.2 at 51.27 cM), an outward rectifying potassium channel (MLOC_18521.1 at 55.1 cM), a vacuolar cation/proton exchanger (MLOC_13658.1 at 51.35 cM), a V-type proton ATPase (AK251977.1 at 51.63 cM), a voltage-gated chloride channel (MLOC_57123.4 at 56.44 cM); and some transcription factors possible involved in guard cell signal transduction such as Myb domain protein MYB (MLOC_7981.1 at 51.63 cM), WD-40 repeat protein (AK370701 at 51.63 cM), Ca^{2+} dependent protein kinase CDPK (MLOC_12765.1 at 47.04 cM), Ca^{2+} independent protein kinase CIPK (MLOC_9827.2 at 49.29 cM) and calcineurin-B like activator CBL (MLOC_60474.3 at 55.17 cM). Although we cannot rule out other genes in this region, the genes listed above are candidates for further fine-mapping and functional analysis to verify their roles in salt tolerance in barley.

5.4 Discussion

5.4.1 Intercellular CO_2 concentration, transpiration rate and stomatal conductance are genetically linked to biomass production in barley

Salinity tolerance in plants including barley is inherently complex, controlled by polygenic traits and is affected by various mechanisms influencing photosynthesis (Chen et al. 2007a; Chen et al. 2005; Chen et al. 2007c). Under control conditions, QTLs for intercellular CO_2 concentration (CI-C), transpiration rate (TR-C), and stomatal conductance (GS-C) were closely located together with that for biomass (BM-C) on chromosome 1H (Figure 5.6, Table 5.1). QTLs associated with photosynthetic traits have rarely been reported in barley due to measurement procedures and the complicated, dynamic processes of these phenotypic traits. Liu et al. (Liu et al. 2015) identified several QTLs located on chromosomes 2H, 3H and 7H associated with intercellular CO_2 concentration, transpiration rate and stomatal conductance

from barley flag leaves on plants grown under normal conditions. QTLs for net photosynthetic rate, transpiration rate and stomatal conductance have also been found in rice (Teng et al. 2004). Therefore, photosynthetic parameters have the potential for selection for salinity tolerance in grasses (Lee et al. 2004). In our experiment, co-localization of QTLs for gas exchange traits with those for biomass production demonstrated that intercellular CO_2 concentration, transpiration rate, and stomatal conductance are genetically linked to biomass production in barley. Moreover, QTLs for relative transpiration rate (TR-T/C) and relative biomass (BM-T/C) were identified at same position on chromosome 2H (Figure 5.6, Table 5.1), suggesting that they are likely to be genetically linked to each.

NaCl-induced accumulation of ABA in leaves leads to stomatal closure and reduced transpiration rate, thereby contributing to increased water use efficiency in plants (Laza et al. 2010). Initial stomatal closure can serve as a rapid and effective response to salinity; however, long-term stomatal closure will limit photosynthetic CO_2 and plant growth (Juenger et al. 2005). CO_2 and water availability strongly influence stomatal opening and closure. Stomatal opening or closure directly affects stomatal conductance which further influences CO_2 intake and transpirational water loss (Kim et al. 2010). Short-term, elevated CO_2 concentrations provoke stomatal closure, whereas long-term, elevated CO_2 concentrations decrease stomatal density leading to reductions in transpiration (Kim et al. 2010; Lake et al. 2002). Stomatal conductance significantly influences net photosynthetic rate and is one of the key parameters limiting photosynthesis in barley (Jiang et al. 2006). In our study, we found genetic evidence for the importance of photosynthetic traits for barley production under salinity stress. Therefore, promoting leaf photosynthetic capacity and genetic manipulation of photosynthesis are important approaches to enhancing crop biomass (Horton 2000).

5.4.2 No linkage among gas exchange and stomatal traits under salinity stress

Gas exchange characteristics depend on stomatal form and structure and are regulated by controlling stomatal aperture and density (Hetherington and Woodward 2003). Salt tolerance was associated with lower stomatal density and decreased stomatal area in *Chenopodium quinoa* (Shabala et al. 2013), and a positive correlation between stomatal frequency and transpiration rate was reported in barley (Miskin et al. 1972). In rice, it was reported that high stomatal density was associated with high photosynthetic rate in *Indica* cultivars, while *Japonica* cultivars had higher transpiration efficiency (Peng et al. 1998). It was suggested that salinity may have a relatively direct impact on the photosynthetic apparatus independent

of that on stomata (Brugnoli and Bjorkman 1992). Moreover, a remarkably negative correlation between stomatal density and size was found in lowland rice but no common QTL was found for these traits (Laza et al. 2010). One of the aims of this study was to find potential QTLs connecting stomatal and photosynthetic traits for salinity tolerance in barley. In this study, only the QTL for stomatal pore area (SA) was associated with transpiration rate (TR), stomatal conductance (GS), or intercellular CO_2 concentration (Figure 5.6). Other QTLs for leaf temperature (LT), vapour pressure deficit (VPD), aperture width/length (AWL) and subsidiary cell length (SCL) showed no links with photosynthetic traits. Therefore, our findings that gas exchange and stomatal traits are not necessarily genetically linked to each other.

5.4.3 QTLs and candidate genes for salinity tolerance score and grain yield

Salinity tolerance, assessed through the combination of plant survival and leaf wilting, has been used for evaluating barley salt tolerance in our previous studies (Fan et al. 2015; Xu et al. 2012; Zhou et al. 2012). Crop yield under salinity stress is a result of balancing resource allocation between growth and defence against stress, since responding to stress is deleterious to growth and yield (Atkinson and Urwin 2012); therefore, the ability to produce high grain yield in saline soils is the ultimate criterion of salinity tolerance. In this study, we identified one QTL controlling salt tolerance (*QST.CmGa.3H*) using plant survival and leaf wilting as an evaluation index. This QTL, located at 60.1 cM on chromosome 3H, explained 16.8 % phenotypic variation. Interestingly, the QTL for grain yield relative to the control (*QGY-T/C.CmGa.3H*) was only 1.2 cM away from the QTL for salinity tolerance (Figure 5.3), indicating that salinity could influence grain yield to some extent. Therefore, we attempt to identify candidate genes for salinity tolerance on chromosome 3H using the published barley genome (Appendix 5.1). These candidate genes included those involved in ROS detoxification, ion transport, and signal transduction. Plants can perceive stress through transmembrane osmo-receptors and transduce the perception of environmental stimuli via internal signalling pathways. Induced transcription factors (TFs) and post-translational regulation of TFs lead to the expression of functional downstream response genes involved in ion channels, secondary metabolite biosynthesis, ROS detoxification, stomatal closure, growth regulation, cell death as well as those encoding late embryogenesis abundant (LEA) proteins (Atkinson and Urwin 2012). Near isogenic lines are being developed in our current research work for fine mapping of these candidate genes.

5.4.4 Co-localisation of QTLs associated with salinity tolerance

Using various genetic populations, some QTLs for salinity tolerance in barley have been found and are associated with chlorophyll content, chlorophyll florescence, proline content, water soluble carbohydrates, relative water content (Siahsar and Narouei 2010), ion content (Nguyen et al. 2013; Xue et al. 2009), and salinity tolerance (Fan et al. 2015; Xu et al. 2012; Zhou et al. 2012). Previous QTL studies of barley salinity tolerance have lacked information on the genetic mechanisms underlying stomatal traits and gas exchange parameters. Interestingly, many QTLs for stomatal traits and gas exchange parameters co-localised with previously identified QTLs for agronomic or physiological traits (Figure 5.4, Table 5.1). The co-localization of QTLs for stomatal conductance, leaf vapour pressure deficit, leaf temperature, chlorophyll content, chlorophyll florescence, water soluble carbohydrate and relative water content with salinity tolerance is not unexpected, because these traits are associated with photosynthesis and transpiration in barley. The relationship among these traits indicates that these parameters may not be independent but interacting. They may be co-regulated for the protection of photosynthetic apparatus, an important factor in tolerance to salinity stress (Munns and Tester 2008). In addition, the co-localisation of QTLs for stomatal conductance and leaf temperature under control or saline conditions could be linked with genes whose proteins control K^+, Na^+ and Cl^- contents. Stomatal opening or closure is controlled by guard cells and adjacent subsidiary cells, and the 'shuttling' of ions and solutes between the two cell types (Mumm et al. 2011) using channels and transporters should be regulated to maintain ionic homoeostasis in these two cell types. The K^+ content in shoots is associated with the shuttle transport of K^+ between subsidiary and guard cells, resulting in prompt stomatal opening and closure. K^+ accumulation was generally detected in subsidiary cells during stomatal closure (Mumm et al. 2011; Raschke and Fellows 1971). K^+ inward and outward rectifying channels and slow anion channels in guard cell could be responsive to ion shuttle transport within the stomatal complex (Chen et al. 2012c; Chen et al. 2010; Wang et al. 2012b). Interestingly, six genes from the published barley genome sequence related with the transport of K^+ and anions are located close to QTL, *QGY-T/C.CmGa.3H*, and to the SSR marker, Bmac0209 (Appendix 5.1). Therefore, the fine mapping of genes encoding K^+ and Cl^- channels should be performed followed by their functional analysis to verify their roles in salinity tolerance in barley.

Chapter 6 Genome-wide association study reveals a new QTL for salinity tolerance in barley (*Hordeum vulgare* L.)

6.1 Introduction

Salinity stress disrupts plant metabolisms, affecting crop yield and restricting the utilization of agricultural land. It has been estimated that 20% of arable land worldwide is salinized which mainly results from natural causes, such as climate change and human influence factors like poor irrigation management (FAO 2008; Flowers and Yeo 1995). At the whole-plant level, salinity stress is considered to be composed of two phases: a rapid osmotic stress which reduces shoot growth, and slower ionic stress which accelerates senescence of older leaves due to elevated leaf Na^+ content (Munns and Tester 2008). Osmotic stress affects plant growth by reducing cell expansion and elongation rates, which leads to smaller and thicker leaves, and down-regulated photosynthesis by reducing stomatal aperture (Bradford 1976). Plants employ numerous mechanisms to adapt to saline conditions. The major ones include Na^+ exclusion from uptake; control of xylem Na^+ loading and/or its retrieval from the shoot; efficient vacuolar Na^+ sequestration; cytosolic K^+ homeostasis and retention in root and mesophyll cells; efficient osmotic adjustment; and ROS detoxification (Munns and Tester 2008; Zhu 2003). Some naturally salt tolerant species such as halophytes also possess a set of unique anatomical features such as salt grands of bladders (Flowers and Colmer 2008; Shabala et al. 2014; Shabala and Mackay 2011). Ion homeostasis is controlled by numerous ion channels, ion sensing and signalling, pathways of transportation and compartmentalization mechanisms (Munns and Tester 2008; Zhu 2003). Since many traits underlying adaption to stress are quantitative and controlled by multiple genetic pathways, a wide variety of genes are implicated in salinity tolerance (DeRose-Wilson and Gaut 2011).

Barley is one of the most important cereal crops worldwide, and also the most salt tolerant cereal (Munns and Tester 2008). Cultivated barley originated from wild barley and domesticated within the Fertile Crescent and Tibet (Badr et al. 2000; Dai et al. 2012; Kilian et al. 2006). Barley is indispensable to malting and brewing industries and also serves as a staple food in some area of the world due to its broad adaption to salinity, drought and high

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altitude (Baik and Ullrich 2008). Both genetic diversity and adaption to broad conditions resulted in a rich gene pool of barley (Nevo and Chen 2010). However, modern cultivated barley varieties only include 15 to 40 % of all alleles within the barley gene pool, indicating that only a small part of barley genetic potential has been used for improvement for salinity tolerance (Ellis et al. 2000; Kilian et al. 2006; Long et al. 2013). Progress in improving crop salinity tolerance or developing salt tolerant cultivars has been lagging behind many improvements in crop biotic stress tolerance due to the fact that salinity tolerance is a physiologically and genetically (quantitative inheritance) complex trait controlled by numerous QTL (Flowers 2004). Traditional bi-parental QTL mapping has been widely used for the dissection of salinity tolerance and the identification of tolerance genes. Bi-parental QTL mapping detects chromosomal regions varying from a few to several tens of centi-Morgans (cM), harbouring a large number of genes (Long et al. 2013). Many QTL for salinity tolerance were detected using a wide variety of agronomic and physiological traits as selection criteria for barley salinity tolerance. These include plant survival (Fan et al. 2015; Xu et al. 2012; Zhou et al. 2012), yield and agronomic traits (Ellis et al. 2002; Xue et al. 2009), seed germination (Witzel et al. 2010), Na^+ exclusion (Shavrukov et al. 2010), tissue ion content (Xue et al. 2009), water soluble carbohydrate and chlorophyll content (Siahsar and Narouei 2010).

Bi-parental QTL mapping has shown the power to identify candidate QTL/genes for salinity tolerance. However, allelic diversity between parents and recombination occurring during the production of populations are limited, which leads to limitations in QTL mapping, although there are some multi-parent populations such as Multi-parent Advanced Generation Inter-Cross (MAGIC) (Korte and Farlow 2013; Kover et al. 2009). Recent rapid development in genotyping and sequencing technologies has enabled novel association mapping to identify alleles in a much broader range of natural accessions. A genome wide association study (GWAS) explores the recombination that has occurred during a long evolutionary history of diverse sets of accessions (Nordborg and Tavare 2002). QTL mapping is suitable for detecting rare alleles of large effect, while GWAS could be a complementary approach for the identification of major allelic variants underlying quantitative and complex traits (DeRose-Wilson and Gaut 2011; Long et al. 2013). Barley has a high level population structure such as two-rowed and six-rowed cultivars, spring and winter barley (Pasam et al. 2012). Due to the confounding effect of population structure, GWAS have a higher chances of producing false positive (type I) and negative (type II) errors than QTL mapping (Zhu et al.

2008). A mixed-linear model (MLM) approach has been developed which leads to a better performance (Yu et al. (2006). In barley, GWAS has been used for detecting genetic variations underlying diverse complex traits such as agronomic and morphologic traits (Cockram et al. 2010; Munoz-Amatriain et al. 2014; Pasam et al. 2012; Wang et al. 2012a), malting quality related traits (Cai et al. 2015; Huang et al. 2014; Matthies et al. 2014), cadmium accumulation (Wu et al. 2015), frost tolerance (Visioni et al. 2013), aluminium tolerance (Cai et al. 2013; Zhou GF et al. 2016) and salinity tolerance (Long et al. 2013).

The objectives of this study were to (1) detect candidate QTL for salinity tolerance in barley through a genome wide association study; (2) and discuss how statistical models affect the power of GWAS. Also, for the first time, we utilized QTL mapping through MapQTL 6.0 software to confirm those QTL detected in GWAS.

6.2 Materials and methods

6.2.1 Barley germplasm and genotyping

A total of 206 barley accessions collected from Europe, Asia, Australia and Canada were used in this study. All the accessions were genotyped with Diversity Arrays Technology (DArT) markers (Wenzl et al. 2004) and distributed over the whole genome. A consensus genetic map was sourced from http://www.diversityarrays.com. More than 1100 polymorphic DArT markers were scored for this population. Among them, 482 markers were found to have a specific chromosome position. A total of 408 markers, with Q value (marker quality) and call rate above 80% as well as minor allele frequency (MAF) higher than 0.05, were used for population structure and association mapping analysis.

6.2.2 Evaluation of salinity tolerance

Salinity tolerance of these barley varieties were evaluated in the 2013 and 2014 barley growing seasons. Experiments were conducted in a glasshouse in Launceston, Tasmania, Australia. Seeds of all the accessions were sown in large plastic containers (1.6 m × 2.5 m × 0.6 m) using a potting mixture described in Fan et al. (2015). Each genotype consisted of three replicates, each of five seedlings. Salt treatment was performed with 300 mM NaCl. A control experiment was not conducted since it had been proved that different varieties, in the same potting mixture without salt added, exhibited no obvious symptoms of leaf chlorosis or

wilting (Zhou et al. 2012). The salt treatment started at the two-leaf stage and was repeated every three days according to our previous method (Xu et al. 2012; Zhou et al. 2012). When the most susceptible lines showed severe symptoms, salinity tolerance was assessed by combining scores for plant survival and leaf chlorosis (0 = no damage and 10 = all dead) (Xu et al. 2012).

6.2.3 Population structure and Kinship analysis

A total of 408 DArT markers distributed over the whole genome were used for population structure analysis using STRUCTURE software (v2.3.3) (Pritchard et al. 2000). The number of clusters (K) was set from 2 to 12 and twenty iterations were conducted in an admixture model with a 10,000 burning period and 10,000 MCMC (Markov Chain Monte Carlo). K value was the number of clusters when ΔK achieved maximum value (Evanno et al. (2005). Principle component analysis (PCA) was performed using GAPIT R package to visualize the dispersion of the association panel in a graph (Lipka et al. 2012). A kinship analysis was conducted using SPAGeDi software (Hardy and Vekemans 2002). The kinship matrix measured the genetic similarity between individuals.

6.2.4 Genome wide association study

A genome wide association study among phenotypic trait (mean value of 2013 and 2014), DArT markers (genotype), population structure and kinship were conducted using TASSEL software (v3.0) (Bradbury et al. 2007). The Q, K and Q+K methods were used for GWAS. For Q model: $y = X\beta + Q\nu + e$; for K model: $y = X\beta + Z\mu + e$; for Q+K model: $y = X\beta + Q\nu + Z\mu + e$. X is DArT marker matrix, Q and Z represent sub-population membership matrix and kinship matrix, respectively, β and ν are coefficient vectors for DArT marker and sub-population membership, respectively, μ is a vector of random genetic effects $\mu \sim N(0, 2K)$ and e is the random error vector. $P < 0.01$ ($-\log_{10}(P) > 2$) was set as the significant threshold in the association study. Manhattan plots were displayed using R software (v2.14.2). For evaluating the fitness and efficiency of different models, quantile-quantile (Q-Q) plots were shown using TASSEL (v3.0).

6.2.5 Confirmation of the number of QTL

A genetic linkage map for this natural population has been constructed using Diversity Array Technology (DArT) markers. The DArT markers consensus genetic map was provided at http://www.diversityarrays.com. The software package MapQTL 6.0 (Van Ooijen 2009) was also used to detect QTL and confirm the relationship between different markers around each QTL, since the genome wide association study resulted in several marker-trait associations with many markers locating at close positions to each other. QTL were first analysed by interval mapping (IM). The marker with highest LOD values at each putative QTL identified using interval mapping was selected as a cofactor and the selected markers were used as genetic background controls in the approximate multiple QTL model (MQM). The population structure (Q-matrix) was used as covariates. A logarithm of the odds (LOD) threshold value of 3.0 was applied to declare the presence of a QTL at 95% significance level.

6.2.6 Genomic analysis of potential genes for salinity tolerance

The nearest marker of the QTL for salinity tolerance, bPb-9668 on 4H (see results), was consistently detected in all methods. bPb-9668 was located at the end of chromosome 4H. Barley genomic data and gene annotations were downloaded from ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/ (Mayer et al. 2012) and ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/popseq_IPK/ (Mascher et al. 2013). Annotated genes within 15cM around bPb-9668 on 4H were examined for potential genes for salinity tolerance.

6.3 Results

6.3.1 Salinity tolerance of barley accessions

Barley accessions exhibited significant difference in their salinity tolerance. Since the scoring was conducted at a relatively early stage of salt treatment when clear phenotypic segregation was shown, most of the sensitive varieties (e.g. Franklin, Gairdner) were scored for 5 while the tolerant varieties (CPI-11284-48, TX9425) had a score of 1. The scores from two years correlated significantly with each other ($r = 0.65$). Therefore, the average data were used for further analysis. Figure 6.1 shows the frequency distribution (the number of accessions) of salinity tolerance based on the average leaf wilting and plant survival scores of all genotypes, ranging from 1 to 8.

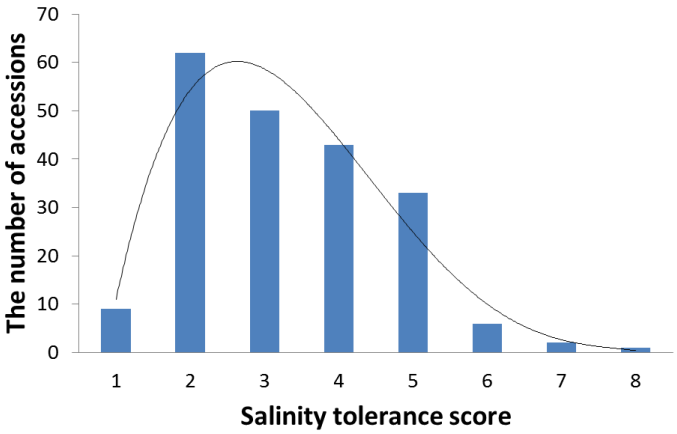

Figure 6.1 The distribution frequency of salinity tolerance scores in 206 barley varieties. Seedlings were treated with 300mM NaCl at the two leaves stage. Salinity tolerance was scored from 0-10 by leaf chlorosis (0 = tolerant, 10 = sensitive). Data were averaged over two growth seasons, 2013 and 2014

6.3.2 Population structure

Cluster parameter K was set from 2 to 12. According to the explanation of Evanno et al. (2005), the largest value of statistic index ΔK was used as an indicator for evaluating the most probable number of subpopulations among all accessions. In this study, ΔK reached the to P value when K = 6 (Figure 6.2). Therefore, the most appropriate number of clusters are represented by six different colours (Figure 6.3). STRUCTURE results were also confirmed by PCA (Figure 6.4). Details of population structures of 206 barley accessions are listed in Additional File (Appendix 6.1).

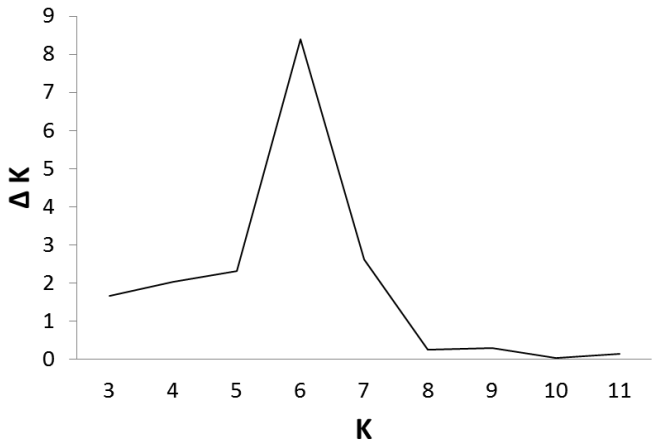

Figure 6.2 An estimation of the most probable number of clusters (K), based on 20 independent runs and K ranging from 2 to 12

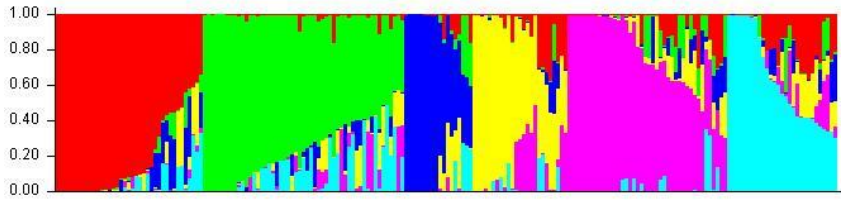

Figure 6.3 The population structure of 206 barley accessions. Six subpopulations (K = 6) were produced based on genetic diversity detected by 408 DArT markers, each are represented by a different colour

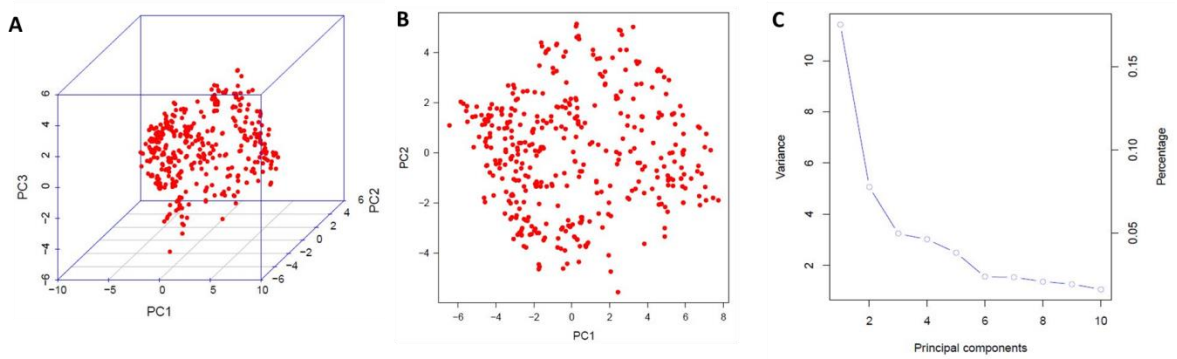

Figure 6.4 Principal component analysis (PCA) of 206 barley accessions. Population structure and dispersion of the association panel were shown through three dimensional (A) and two dimensional (B) diagrams. (C), Number detection of subpopulations or principal components.

6.3.3 Association mapping for salinity tolerance

Salinity tolerance of 206 barley accessions and 408 DArT markers were used for association mapping. A total of 24 significant marker-trait associations were detected with Q method. These markers are located on 2H, 3H, 4H, 5H, 6H and 7H (Figure 6.5, Table 6.1), representing 12 potential QTL. Only two significant marker-trait associations (one QTL) were detected on 4H with the K method, while two significant marker-trait associations representing two QTL were identified with the Q+K method, located on 2H and 4H, respectively (Figure 6.5, Table 6.1).

Quantile-quantile (Q-Q) plot was employed to evaluate the fitness and efficiency of different models. The observed $-\log_{10}$ (P) values for salinity tolerance were closer to expected $-\log_{10}$

(P) values from the K and Q+K methods than those from the Q method (Figure 6.6). However, only one or two QTL were detected in the K or the Q+K methods, whereas about 12 QTL were detected with the Q method.

Table 6.1 Association mapping results for salinity tolerance with the Q method, K method and Q+K method respectively (P < 0.01).

Method	Trait	Chromosome	Position	Marker	P	Marker R^2
Q+K (MLM)	SLAV	2H	25.7	bPb-0003	0.0060	0.046
	SLAV	4H	145	bPb-9668	0.0091	0.038
K (MLM)	SLAV	4H	145	bPb-9668	0.0018	0.049
	SLAV	4H	145.1	bPb-5265	0.0041	0.041
Q (GLM)	SLAV	2H	3.5	bPb-5489	0.0002	0.059
	SLAV	2H	3.5	bPb-4285	0.0002	0.056
	SLAV	2H	5	bPb-5191	0.0002	0.056
	SLAV	2H	5.3	bPb-9681	0.0005	0.056
	SLAV	2H	25.7	bPb-8399	0.0006	0.056
	SLAV	2H	25.7	bPb-0003	0.0008	0.049
	SLAV	2H	35.7	bPb-1196	0.0098	0.028
	SLAV	3H	20	bPb-6978	0.0037	0.036
	SLAV	3H	97.4	bPb-6722	0.0055	0.032
	SLAV	3H	145.5	bPb-4156	0.0052	0.033
	SLAV	3H	145.5	bPb-5298	0.0067	0.032
	SLAV	3H	145.5	bPb-5396	0.0068	0.031
	SLAV	4H	145	bPb-9668	0.0017	0.043
	SLAV	4H	145.1	bPb-5265	0.0020	0.040
	SLAV	5H	43.5	bPb-4135	0.0081	0.030
	SLAV	5H	97.9	bPb-2425	0.0024	0.039
	SLAV	5H	98.2	bPb-8101	0.0013	0.044
	SLAV	5H	166.1	bPb-6179	0.0042	0.035
	SLAV	5H	168.3	bPb-0835	0.0042	0.035
	SLAV	5H	168.3	bPb-4595	0.0042	0.035
	SLAV	5H	173.7	bPb-1719	0.0087	0.029
	SLAV	6H	38	bPb-2058	0.0093	0.029
	SLAV	6H	68.2	bPb-5698	0.0034	0.036
	SLAV	7H	140.9	bPb-5923	0.0072	0.031

*SLAV: Salinity tolerance data are averaged over two growth seasons 2013 and 2014 based on combined scores of plant survival and leaf chlorosis.

P value < 0.01 have been used as a cut-off for barley GWAS in many studies (Huang et al. 2014; Pasam et al. 2012; Shu et al. 2012). The marker bPb-9668 on 4H (145.0 cM) showed consistent significance (P < 0.01) of marker-trait associations using the Q, K and Q+K methods (Figure 6.5, Table 6.1). Another marker, bPb-5265 (145.1 cM) on 4H which is close to bPb-9668, showed significance under the Q and K methods, not Q+K method (Table 6.1). The marker bPb-0003 on 2H showed significant marker-trait associations with both the Q and the Q+K methods but not the K method (Figure 6.5, Table 6.1).

Based on marker polymorphisms, the salinity tolerance of 206 barley accessions was grouped into two genotypes according to their base calls of the marker bPb-9668 and bPb-0003 (Figure 6.7A; Appendix 6.2). Accessions with different polymorphisms at bPb-9668 and bPb-0003 showed highly significant differences in salinity tolerance (P < 0.0001, Figure 6.7A; Appendix 6.2).

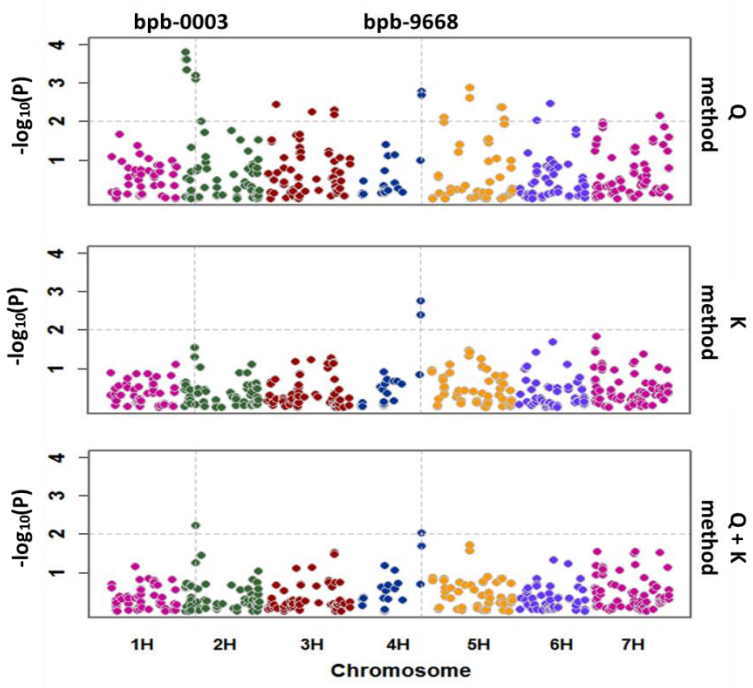

Figure 6.5 A Manhattan plot for genome wide association study (GWAS) of salinity tolerance in 206 barley accessions. GWAS was analysed by three methods: (A), Q method; (B), K method; (C), Q + K method. Significant associations were identified using criterion of $-\log_{10}(P) > 2$ ($P < 0.01$)

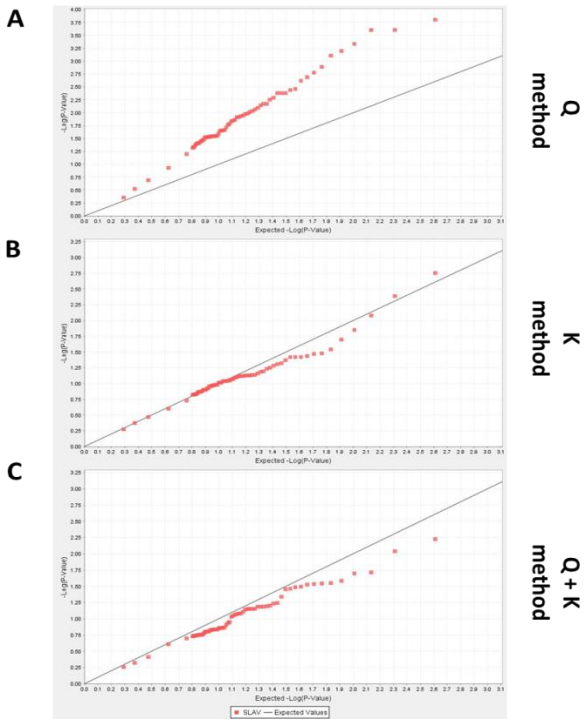

Figure 6.6 Quantile–quantile (Q-Q) plots of estimated -$\log_{10}$(P). Q-Q plots were displayed in marker-trait association analysis using three models: (A) Q method; (B) K method; (C) Q+K method. The black line represents the expected line under the null distribution. The red symbol is the observed -$\log_{10}$(P) for salinity tolerance

6.3.4 QTL mapping for salinity tolerance using MapQTL6.0 software

Many marker-trait associations were detected using the Q-method with some of them being located at similar positions. In order to identify the similarity of those markers located at similar positions, MapQTL6.0 was used to detect significant QTL. When analysed for QTL using MapQTL6.0 software using population structure (Q-matrix) as covariates, the results were very close to association mapping with the Q method (Figure 6.8). The analysis produced 3 significant QTL (LOD > 3.0) and 4 tentative QTL (3 > LOD > 2) (Table 6.2), with all of them being in line with those from association mapping with the Q method. The most significant QTL on 4H was the same as that identified with both the K and the Q+K methods (Tables 6.1 and 6.2). MQM mapping resulted with, apart from two QTL based on bPb-9668 and bPb-0003 from the K or the Q+K method (Figure 6.5; Table 6.1), two more QTL with nearest marker bPb-4285 and bPb-4135 being significant with LOD > 3.0 (Table 6.2). bPb-4285 also showed a highest –$\log_{10}$(P) in the Q method (Figure 6.5; Table 6.1). The salinity tolerance of the 206 barley accessions was also grouped into two genotypes

according to the base calls of the marker. As shown in Figure 6.7A and Appendix 6.2, all four markers showed significant association with salinity tolerance. The four QTL showed additive effects with the average salinity tolerance being increased with the increased number of tolerance alleles. The average damage score of varieties combining all four tolerance alleles was 2.1, while that of no tolerance alleles was 5.2 (Figure 6.7B; Appendix 6.2).

Table 6.2 QTL mapping results for salinity tolerance in 206 barley varieties when structure was used as covariate (LOD > 2.0).

Trait	Chromosome	Position	Locus	LOD	R^{2*}
SLAV**	2H	3.5	bPb-4285	3.66	4.8
SLAV	2H	25.7	bPb-0003	2.11	2.6
SLAV	3H	133.5	bPb-6504	2.12	2.7
SLAV	4H	145	bPb-9668	5.67	7.5
SLAV	5H	43.5	bPb-4135	3.91	5.1
SLAV	7H	3.5	bPb-3732	2.13	2.6
SLAV	7H	125.4	bPb-8539	2.47	3.2

*R^2: the percentages of phenotypic variation explained by markers
**SLAV: Salinity tolerance data are averaged over two growth seasons 2013 and 2014

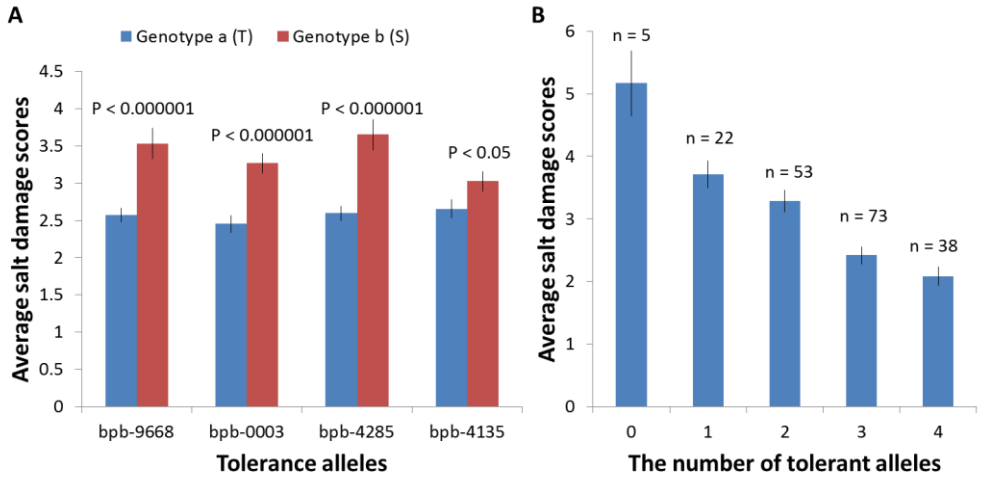

Figure 6.7 Salinity tolerance of 206 barley accessions of two genotype groups based on their Base calls of the markers: bPb-9668, bPb-0003, bPb-4285 and bPb-4135. (A), Accessions with different polymorphisms at these four markers showed very significant differences in salinity tolerance; *T: tolerant, S: sensitive. (B), These four QTL showed an additive effect with the average tolerance score (2.08) of varieties combining all four tolerance alleles than that of varieties with all susceptible alleles (5.167); *0: without any tolerance alleles, 1-3: with 1-3 tolerance alleles, 4: with all four tolerance alleles

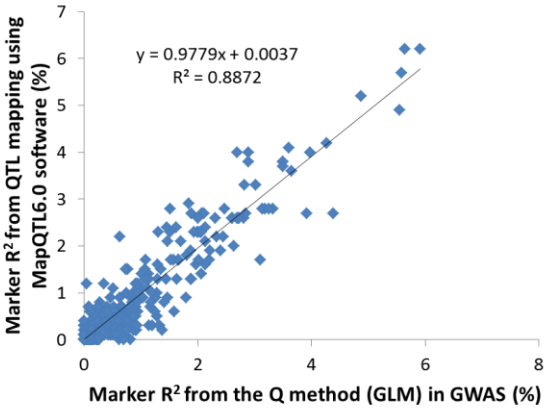

Figure 6.8 Correlations between marker R^2 from the Q method (GLM model) in GWAS and the R^2 from QTL mapping using MapQTL6.0 software with population structure (Q-matrix) as covariates. R^2: the percentages of phenotypic variation explained by markers

6.3.5 Potential genes for salinity tolerance on 4H

In this study, QTL on 4H with the nearest marker of bPb-9668 was the most significant, consistently detected in all methods. Annotated genes around this marker on 4H are listed in Appendix 6.3. Among all annotated genes, there are two possible genes likely to be associated with salinity tolerance, MLOC_70918.1 and MLOC_5021.1. Both locate at the end of chromosome 4H and close to the marker bPb-9668 according to the POPSeq map (Mascher et al. 2013). MLOC_70918.1 belongs to glutathione-regulated potassium-efflux system protein while MLOC_5021.1 is a respiratory burst oxidase-like protein. Ion homeostasis, especially Na^+ and K^+, are related to salinity tolerance (Munns and Tester 2008). *RESPIRATORY BURST OXIDASE HOMOLOG F* (*RBOHF*) encodes a specific isoform of NADPH oxidase, which plays a vital role in soil salinity tolerance (Jiang et al. 2012).

6.4 Discussion

6.4.1 A new QTL for salinity tolerance was identified by association mapping

Salinity tolerance is a genetically and physiologically complex trait controlled by numerous QTL (Flowers 2004). Leaf wilting and plant survival are two of the major symptoms caused by salt stress and had been used for evaluating salinity tolerance of barley through traditional bi-parental QTL mapping in many studies (Fan et al. 2015; Xu et al. 2012; Zhou et al. 2012). In the present experiments, 206 barley accessions were assessed for salinity tolerance and various mapping methods were used to identify QTL controlling salinity tolerance. Different

numbers of QTL were identified using different mapping methods. Association mapping using Q methods identified 12 QTL which are located on 2H (3.5, 25.7 cM), 3H (20, 97.4, 145.5 cM), 4H (145 cM), 5H (43.5, 97.9, 166.1 cM), 6H (38, 68.2 cM) and 7H (140.9 cM), respectively (Figure 6.5, Table 6.1). Most of these QTL were further confirmed by analysing QTL using MapQTL 6.0 software. Some of them were located at similar positions to those reported before by GWAS or bi-parental QTL mapping. The QTL on 2H located at a similar position to that reported in the DH population of TX9425/Naso Nijo (Xu et al. 2012). QTL on 6H with the nearest marker bPb-2058 was close to QSl.Yy.Fr.6H (26 cM) from the DH population of YYXT and Franklin (Zhou et al. 2012). No QTL was reported for salinity tolerance on 4H at the same position (145 cM) of the QTL identified by all four methods in the current study. The nearest QTL for a salinity tolerance-related trait on 4H was located at 119.1 cM in their consensus map (Close et al. 2009), controlling shoot Na^+/K^+ under saline conditions (Long et al. 2013), which is also close to the telomere of 4HL.

6.4.2 GWAS results are affected by models and evaluation methods

In this study, GWAS was conducted with three different models, Q (population structure), K (kinship) and Q+K. According to the Q-Q plots (Figure 6.6), K and Q+K were similar, and both stricter than the Q model. The observed $-\log_{10}$ (P) values for salinity tolerance deviated from the expected $-\log_{10}$ (P) values in the Q method (general linear model), indicating that they may contain false positive associations (Figure 6.6A). The addition of genetic relatedness (i.e. relationship or kinship) makes a mixed-linear model more powerful, thus reducing the number of false positive associations (Yu et al. 2006). K and Q+K were similar in this study on the basis of the Q-Q plots and results in Table 6.1, which was in accordance with Cai et al. (2013). However, only two and one QTL were identified with the K method and the Q+K method, respectively, while a lot more QTL were identified with the Q method. Therefore, QTL mapping was also conducted with the MapQTL 6.0 software (Van Ooijen 2009) using population structure as covariate to adjust the natural variations of this population. Nearly all the QTL identified with MapQTL6.0 were in line with those from association mapping with the Q method and the most significant one was the same as that identified using the K and Q+K methods. The percentages of phenotypic variation explained by various markers analysed with MapQTL 6.0 are very close to those analysed with Q method ($R^2 = 0.89$, Figure S3).

To compare the robustness of combining different mapping approaches, all the accessions were grouped based on their base calls of the markers bPb-9668, bPb-0003, bPb-4285 and bPb-4135 (Figure 6.7; Appendix 6.2), the four significant QTL detected with both GWAS (Q method) and MapQTL 6.0 (Table 6.1 and 6.2). Accessions with different polymorphisms at bPb-9668, bPb-0003, bPb-4285 and bPb-4135 had differences in tolerance scores of 0.960 ($P < 0.000001$), 0.814 ($P < 0.000001$), 1.053 ($P < 0.000001$) and 0.371 ($P < 0.05$), respectively (Appendix 6.2). These four QTL also showed additive effects with the average tolerance score (2.1) of varieties combining all four tolerance alleles being significantly better than that of varieties with all susceptible alleles (5.2) (Figure 6.7; Appendix 6.2). There could be higher chances of false positive or negative errors in GWAS than in bi-parental QTL mapping, resulting from the complex population structure (Myles et al. 2009; Pasam et al. 2012), thus the MLM approach using the K matrix or a combination (Q+K) could perform better than GLM. However, in this study, the K or the Q+K methods were shown to be too strict, resulting in the missing of some possibly useful QTL. QTL mapping using MapQTL6.0 with the Q matrix as covariates in natural populations showed similar power as GLM. The advantage of using the MQM of MapQTL 6.0 is the confirmation of the number of QTL through cofactor selection (Van Ooijen 2009).

6.4.3 Confirmation of QTL identified by GWAS

Salinity tolerance is a quantitative trait controlled by many QTL. Many methods have been used to identify the QTL. Care should be taken to balance the rate of false positives and negatives during the process of analysis using different models/methods (Pasam et al. 2012). Traditional QTL mapping through bi- or multi-parental populations is a powerful method but suffers from a limited amount of recombination. GWAS can partly overcome the limitation by using a diverse germplasm but may lead to a number of false positive or negative associations. Different methods can be complementary to each other and benefit can be achieved by mitigating the other's limitations (Korte and Farlow 2013). In this study, the combination of GWAS and QTL mapping has led to successful identification of QTL with potential application in breeding programs. However, the QTL identified by GWAS requires further confirmation in bi- or multi- parental populations.

Conclusions

In this study, twenty-four markers showed significant association with salinity tolerance. Different methods were used for QTL detection concluding with four significant QTL. These QTL showed additive effects with salinity tolerance being greatly increased by combining all four tolerance alleles. A new QTL on 4H (telomere of the long arm) was detected with different methods and will be further investigated. Overall, the K or the Q+K method was stricter than the Q method but may result in some missed useful QTL. The Q method, with similar power as MapQTL 6.0 using population structure as covariate, discovered more QTL but could have produced false positives. Population size, accuracy of phenotyping, and quantity of markers can be increased to enhance the power of association mapping, and further confirmation of QTL will be needed. The confirmed QTL can then be used in breeding programs.

Chapter 7 Fine mapping of a major QTL on 2H for salt tolerance in barley

7.1 Introduction

Soil salinization is a growing problem due to global climate changes and many irrigation practices (Deinlein et al. 2014; Rengasamy 2010). Soil salinity is a major environmental constraint to crop yield and loss of arable land, while the world population is rapidly expanding which needs more crop production (Deinlein et al. 2014). The ultimate aim of salinity tolerance research is to breed salinity tolerant crops and decrease the effects of salinity stress on plant yield (Roy et al. 2014). Diverse biotechnologies can facilitate this by speeding gene discovery and delivering the salinity tolerance genes to cultivate crops through conventional breeding, marker assisted selection (MAS) or genetic modification (Roy et al. 2014). Successful breeding with MAS relies on the selection of appropriate molecular markers. Therefore, fine mapping is needed to find reliable markers not only for MAS and QTL pyramiding, but also benefits map-based cloning for genes discovering.

Numerous genes/locus have been discovered responsible for salinity tolerance in many plants, mostly focused on ion transporters. Studies of model plants (mainly Arabidopsis) have identified *SOS1* (salt overly sensitive1) and *NHX* (Na^+/H^+ exchanger) as key determinants of Na^+ homeostasis. Na^+/H^+ exchanger SOS1 is responsible for the efflux of Na^+ across the plasma membrane, while the vacuolar Na^+/H^+ antiporter NHX1 transports cytosolic Na^+ into vacuoles across the tonoplast membrane (Apse and Blumwald 2007; Yamaguchi and Blumwald 2005; Zhu 2001, 2002). Overexpression of *SOS1*, *NHX1* exhibited increased salinity tolerance in plants (Yamaguchi and Blumwald 2005). AVP1, an H^+-pyrophosphatase (H^+-PPase), also contributes to salinity tolerance. Overexpression of H^+ pump *AVP1* leads to enhanced plant salinity tolerance (Gaxiola et al. 2001; Park et al. 2005; Yang et al. 2007). In rice, a major QTL (*Saltol*), also known as *SKC1*, was identified for shoot K^+ content in seedlings (Lin et al. 2004). Further fine mapping of this QTL revealed that it encodes a HKT-type transporter, named as *OsHKT1;5*, which locates at plasma membrane and is preferentially expressed in the parenchyma cells surrounding the xylem vessels (Ren et al. 2005). *SKC1* (*OsHKT1;5*) contributes to K^+/Na^+ ratio in the shoots, possibly through unloading Na^+ from xylem (Ren et al. 2005). In wheat, two major genes, *Nax1* and *Nax2* (*TmHKT1;4-A* and *TmHKT1;5-A*), were identified for Na^+ exclusion in durum wheat (James

et al. 2011; James et al. 2012). *Nax1* is responsible for Na^+ partitioning from xylem vessels into leaf sheaths, leading to low Na^+ blade: sheath ratio and reduced leaf Na^+ concentration (Huang et al. 2006; Lindsay et al. 2004). According to comparative mapping of wheat and rice chromosomes, a sodium transporter (*TmHKT1;4-A*) suggested to be a strong candidate gene for *Nax1* (Huang et al. 2006). *Nax2* encodes a Na^+-selective transporter on the plasma membrane of root cells surrounding xylem vessels, which confers a reduced transport of Na^+ to leaves through withdrawing Na^+ from the root xylem (Munns et al. 2012). *Nax2* is very similar to another locus *Kna1* in bread wheat (James et al. 2011). *Kna1* (*TaHKT1;5-D*) was identified which contributes to a lower Na^+/K^+ ratio and higher salt tolerance in bread wheat (Dubcovsky et al. 1996). In barley, a locus controlling shoot sodium exclusion, *HvNax3*, was identified from the QTL analysis using a cross between the wild barley (*Hordeum vulgare ssp. spontaneum*) accession CPI-71284-48 and a cultivated barley (*H. vulgare ssp. vulgare*) cultivar Barque-73 (Shavrukov et al. 2010). *HvNax3* locus was delimited to a 0.4 cM genetic interval, where *HVP10* gene encoding vacuolar H^+-pyrophosphatase (V-PPase) is a prime candidate for this locus (Shavrukov et al. 2013). In addition, two allelic variants of *HKT* genes (*HvHKT1* and *HvHKT2*) were evaluated for the allelic function in Tibetan wild barley (Qiu et al. 2011). Allelic variations in the gene of interest can be used for designing markers for salinity tolerance, and can also provide novel sources of genetic material for MAS (Roy et al. 2014).

In our previous study, a major QTL for salinity tolerance was identified from the cross of TX9425 and Naso Nijo (Xu et al. 2012). Although several genes or locus have been discovered to be responsible for salinity tolerance, very few genes were positionally cloned from QTL mapping where some scientists pick the best candidate from a big interval. In the current study, we further fine mapped this QTL, and also developed near isogenic lines based on the new developed marker, which can be used for marker assisted selection in barley breeding and benefit the discovery of candidate gene.

7.2 Methods and Materials

7.2.1 Plant materials and mapping population

A total of 188 F_1-derived double haploid (DH) lines were produced from the cross between TX9425 and Naso Nijo. TX9425 is a Chinese landrace barley variety tolerant to salinity, while Naso Nijo is a Japanese malting barley variety which is sensitive to salinity (Fan et al.

2015; Xu et al. 2012). In our previous study, 551 DArT markers and 75 SSR markers have been used for map construction and one major QTL was discovered controlling salinity tolerance on chromosome 2H.

7.2.2 The development of new molecular markers

New Indel (insertion and deletion) markers were developed according to the method described by Zhou et al. (2015). Closest marker around the QTL for salinity tolerance detected in our previous work was used for BLAST analysis of barley genome DNA sequences at http://webblast.ipk-atersleben.de/barley/viroblast.php. Genomic DNA sequences of three barley cultivars (Morex, Barke and Bowman) were obtained from ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/. Sequences of Morex contig in the region of our interest were used to blast with Barke and Bowman sequence. Alignments among the sequences of three varieties were conducted to explore Indel using the software Genieous. Primers were designed based on these Indel. Those primers with polymorphism between parents (TX9425 and Naso Nijo) were used for fine mapping of the QTL.

7.2.3 Construction of near isogenic lines (NILs) and phenotyping

Two pairs of DH lines with contrast salinity tolerance were selected for crossing. These two lines also have similar genetic background except for the QTL region. NILs were produced through self-breeding in glasshouse. Firstly, embryos of seeds before them turning yellow were collected for culturing in MS medium. After 3 days of culture under dark and 7 days under light, seedlings were transplanted to potting mix in glasshouse. Heterozygotes of QTL region was screened by the nearest Indel marker from F_1 to F_5 through marker assisted selection, while several SSR markers distributed the whole genome were used to select homozygotes in other area except QTL area for ‘purifying’ the background.

Salinity tolerance was evaluated among F_6 plants with two different homozygous genotypes (with TX9425 and Naso Nijo allele, respectively). 320 mM NaCl solution was applied from two and half leaves stage. According to the results of three independent salinity tolerance evaluation, two pairs of NILs with contrast salinity tolerance were selected for further analysis.

7.2.4 Genotyping of parents, DH lines and two pairs of NILs

Genomic DNA was extracted from the leaf tissue of four-week old seedlings, according to the plant DNA extraction protocol for DArT (https://www.diversityarrays.com/files/DArT_DNA_isolation.pdf). Two parental varieties, two pairs of NILs and 188 DH lines were genotyped with DArTSeq (http://www.diversityarrays.com/dart-application-dartseq). A total of 28047 DArT and 8928 SNP markers were used for genotyping, with 3265 DArT and 693 SNP on 2H. After removing markers with greater distortion and missing data, 4788 markers were chosen for map construction.

7.2.5 Map construction of DH population and two pairs of NILs

A new genetic map of the DH population was conducted using the software package JoinMap 4.0 (Van Ooijen 2006). QTL analysis was conducted by the software package MapQTL 6.0 (Van Ooijen 2009). Interval mapping (IM) was firstly used to detect the major QTL. The nearest marker at the QTL from IM was chosen as a cofactor in the multiple QTL model (MQM). Linkage map showing the QTL positions was generated using MAPCHART (Voorrips 2002). In addition, the genetic map of Morex contigs from next-generation sequencing (NGS) (ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/) was from the POPSEQ genetic map (Mascher et al. 2013). Genetic map of two pairs of NILs was conducted by MAPCHART (Voorrips 2002).

7.3 Results

7.3.1 Fine mapping with new developed markers

A major QTL for salinity tolerance was detected in the previous study using DH population from the cross of TX9425 and Naso Nijo, with bpb-6792 as nearest marker which explained 45% phenotypic variation (Xu et al. 2012). To further fine map the gene, we developed several Indel markers (19, 2086-5, 65, 103, 6792-1, 3459-6, 7626-3) according to the physical and functional sequence assembly of barley cultivar Morex (Mayer et al. 2012). Meanwhile, all DH lines were genotyped with high-throughput Diversity Arrays Technology (DArT) markers and SNP markers. A new genetic map was constructed which integrated new developed and high throughput markers (Indel, DArT, SNP) into previous map (Xu et al.

2012). The new developed Indel marker 6792-1 becomes the closest marker for this QTL (Figure 7.1), which explains 67% phenotypic variation (LOD: 36.2).

In addition, according to three recombinant lines (T83074, T83019, T83045), the QTL was delimited to a 0.8 cM interval with Indel marker 19 (6.6 cM) and DArT marker 3268228d (7.4 cM) as flanking markers (Table 7.1). These three lines are all salinity tolerant, and target gene should be localised within the area between Indel marker 19 and DArT marker 3268228d which displayed Naso Nijo (sensitive) genotypes. Markers in Table 7.1 had already been adjusted according to the POPSEQ genetic map (Mascher et al. 2013).

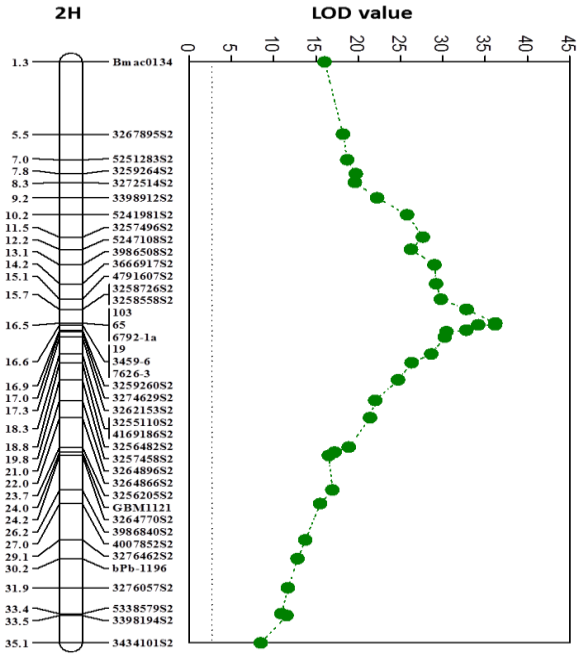

Figure 7.1 QTL for salinity tolerance on 2H. Green dotted line shows the LOD value of markers, with highest LOD value of 36.2 at marker 103, 65 and 6792-1. Black dashed line represents the level of significance (LOD = 3.0).

Table 7.1 Genotyping of recombinant DH lines, near isogenic lines and parents within the genetic target interval (2H, 6.6 cM - 9.4 cM)

Markers	Genotype									Morex WGS contig	Genetic position (POPSEQ)
	T83074	T83019	T83045	T46	N33	T66	N53	TX9425	Naso Nijo		
3809826s	N	N	N	T	N	T	N	T	N	morex_contig_82152	2H, 6.6 cM
3663452d	N	T	T	T	N	T	N	T	N	morex_contig_178235	2H, 6.6 cM
19	N	T	T	T	N	T	N	T	N	morex_contig_178235	2H, 6.6 cM
2086-5	T	T	T	T	N	T	N	T	N	morex_contig_1567230	2H, 6.6 cM
65	T	T	T	T	N	T	N	T	N	morex_contig_1586123	2H, 7.2 cM
103	T	T	T	T	N	T	N	T	N	morex_contig_1577898	2H, 7.2 cM
5257498d	T	T	T	T	N	T	N	T	N	morex_contig_6430	2H, 7.2 cM
3268336d	T	T	T	T	N	T	N	T	N	morex_contig_2546650	2H, 7.2 cM
6792-1	T	T	T	T	N	T	N	T	N	morex_contig_369183	2H, 7.4 cM
4783228d	T	T	T	T	N	T	N	T	N	morex_contig_41429	2H, 7.4 cM
4782858d	T	T	T	T	N	T	N	T	N	morex_contig_50954	2H, 7.4 cM
3259312d	T	T	T	T	N	T	N	T	N	morex_contig_58323	2H, 7.4 cM
3268228d	N	N	N	T	N	T	N	T	N	morex_contig_1564612	2H, 7.4 cM
3459-6	N	N	N	T	N	T	N	T	N	morex_contig_132344	2H, 7.8 cM
3266106d	N	N	N	T	N	T	N	T	N	morex_contig_58109	2H, 7.8 cM
3255389s	N	N	N	T	N	T	N	T	N	morex_contig_1564856	2H, 7.8 cM
3273101s	N	N	N	T	N	T	N	T	N	morex_contig_58728	2H, 7.8 cM
3272804d	N	N	N	T	N	T	N	T	N	morex_contig_64336	2H, 7.9 cM
3665253s	N	N	N	T	N	T	N	T	N	morex_contig_7118	2H, 7.9 cM
7626-3	N	N	N	T	N	T	N	T	N	morex_contig_101967	2H, 8.3 cM
3266250d	N	N	N	T	N	T	N	T	N	morex_contig_101967	2H, 8.3 cM
4006741s	N	N	N	T	N	T	N	T	N	morex_contig_1566867	2H, 8.6 cM
3265235d	N	N	N	T	N	T	N	T	N	morex_contig_66123	2H, 8.6 cM
3270182d	N	N	N	T	N	T	N	T	N	morex_contig_48709	2H, 8.9 cM
3255789s	N	N	N	T	N	T	N	T	N	morex_contig_39078	2H, 8.9 cM
3263495d	N	N	N	T	N	T	N	T	N	morex_contig_135181	2H, 8.9 cM
3264677d	N	N	N	T	N	T	N	T	N	morex_contig_159088	2H, 8.9 cM
3257458s	N	N	N	T	N	T	N	T	N	morex_contig_78699	2H, 9.2 cM
3265251d	N	N	N	T	N	T	N	T	N	morex_contig_46929	2H, 9.3 cM
3433755d	N	N	N	T	N	T	N	T	N	morex_contig_274347	2H, 9.3 cM
3986360d	N	N	N	T	N	T	N	T	N	morex_contig_127084	2H, 9.3 cM
3254734d	N	N	N	T	N	T	N	T	N	morex_contig_1575943	2H, 9.4 cM

*T83074, T83019 and T83045 are recombinant lines from DH population;
T46, N33 and T66, N53 are two pairs of near isogenic lines; TX9425 and Naso Nijo are two parents;
*Tolerant lines: T83074, T83019, T83045, T46, T66, TX9425; Sensitive lines: N33, N53, Naso Nijo;
*d: DArT markers; s: SNP markers;
*T: TX9425 type (tolerant), N: Naso Nijo type (sensitive).

7.3.2 Near isogenic lines exhibited contrasting salinity tolerance

To target the salinity gene more accurately and eliminate the noise caused by other genetic background, near isogenic lines were developed based on Indel marker 6792-1. Heterozygotes at 6792-1 were selected for every generation from F_1 to F_5. Salinity tolerance was evaluated among homozygotes in F_6 generations. Two pairs of NILs were chosen since they exhibited similar morphology (Figure 7.2A) but contrast salinity tolerance (Figure 7.2B, C, D). Each pair of NIL comes from one heterozygote at 6792-1 in F_5. As can be seen from figure 2, lines with TX9425 genotype at 6792-1 (T46, T66) exhibited better salinity tolerance than corresponding lines with Naso Nijo genotype at 6792-1 (N33, N53) at both early and late stage of salt treatment. CM72 and Gairdner were selected to be used as salinity tolerance and sensitive controls when evaluating the tolerance of NILs (Figure 7.2B, C, D). Sensitive lines (N33, N53) and Gairdner displayed very severe leaf chlorosis at first (Figure 7.2B, C), and the whole plant died ultimately (Figure 7.2D).

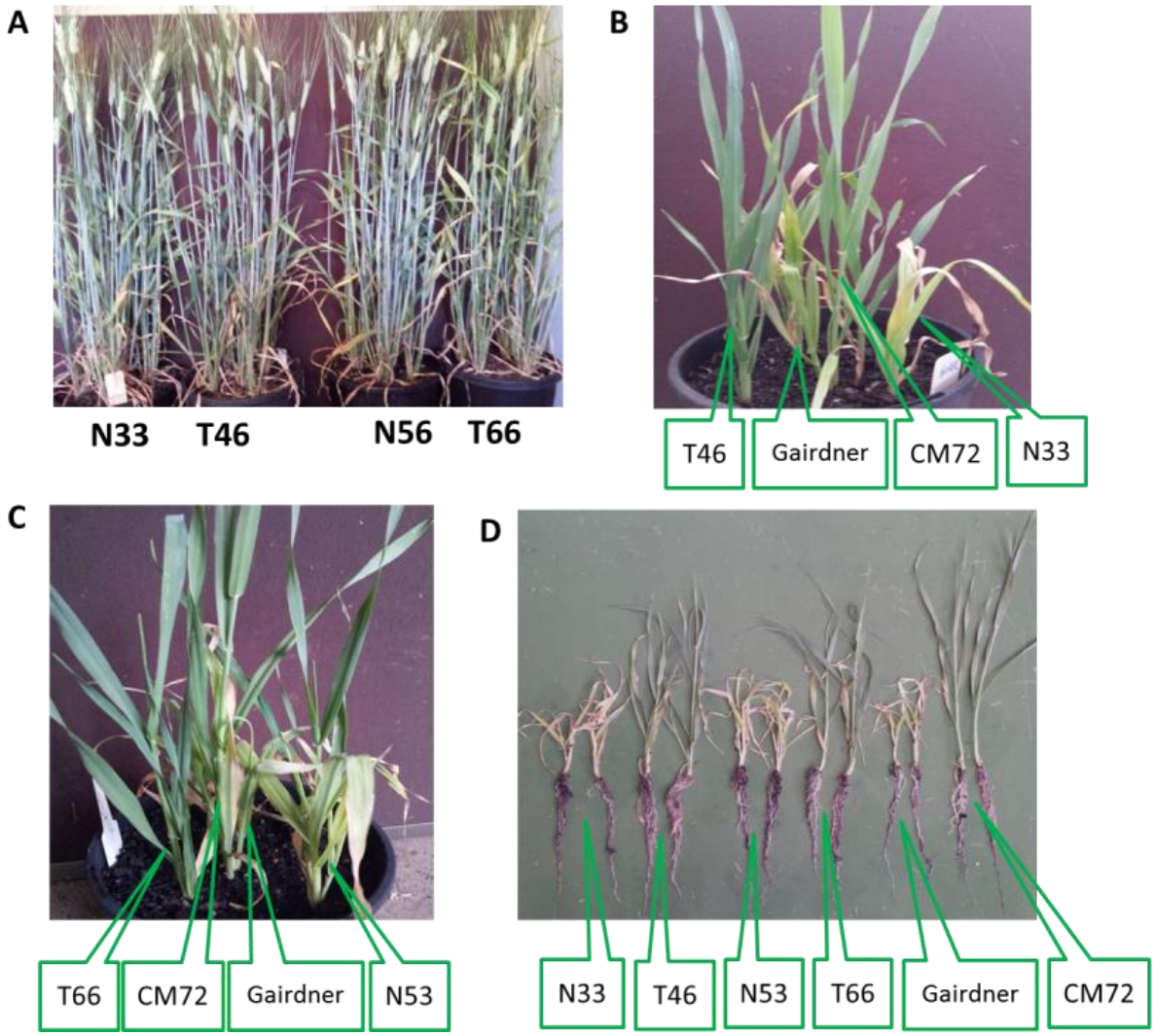

Figure 7.2 Morphology and salinity tolerance of two pairs of near isogenic lines (T46/N33, T66/N53). Each pair of NIL exhibits same growth morphology (A). Two pairs of NILs were treated with 320 mM NaCl at growth stage (two and half leaves). T46 and T66 showed better salinity tolerance than N33 and N53 at both early stage (B, C) and late stage (D). T: TX9425-type at marker 6792-1, salt tolerant; N: Naso Nijo-type at marker 6792-1, salt sensitive.

High through-put genotyping was also conducted for these two pairs of NILs and parents (TX9425/Naso Nijo). Genotypes of all chromosomes were compared among these NILs (Figure 7.3). For both pairs of NILs, there was a different region around 6792-1 in the target area of our interest on chromosome 2H. After adjusting based on the new released physical map of morex contig and the POPSEQ genetic map, this region spans from 6.6 to 9.4 cM which has been indicated by yellow circle in Figure 7.3. However, there is another area which showed difference at the end of chromosome 6H in the pair of T46/N33. Morex contigs and

genetic map positions (POPSEQ) of these two pairs of NILs on chromosome 2H were shown in Table 7.1. Overall, there are 2.8 cM (6.6 to 9.4 cM) intervals existing in these NILs on chromosome 2H.

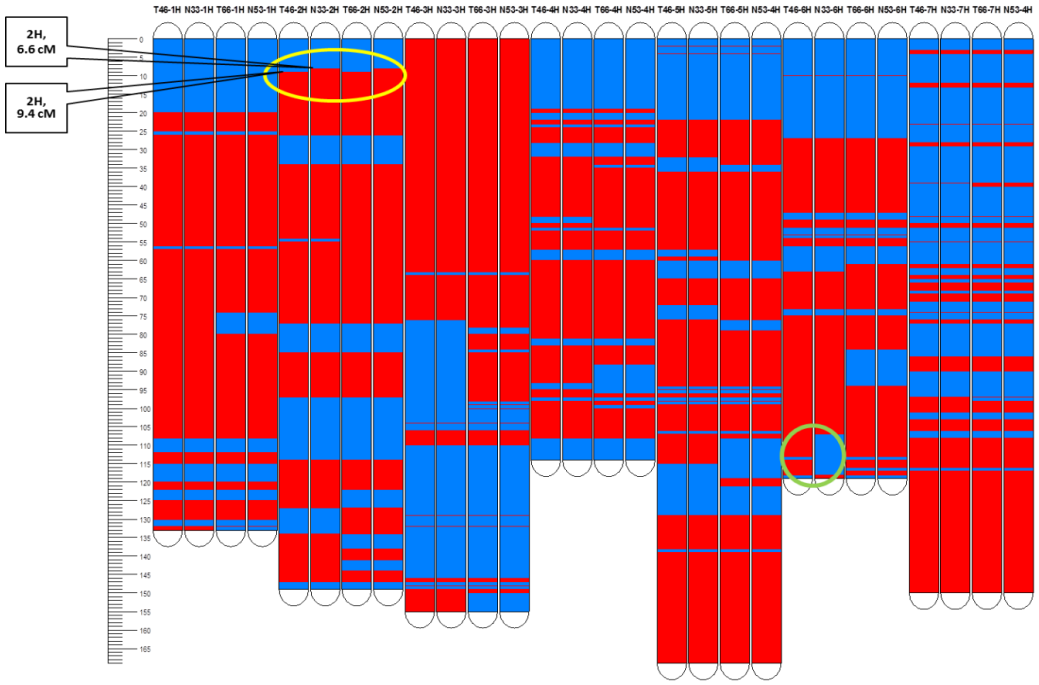

Figure 7.3 Comparison between genotypes of near isogenic lines (two pairs: T46/N33, T66/N53) from the cross of TX9425/Naso Nijo. Red: Naso Nijo alleles; blue: TX9425 alleles. The major difference is on 2H at the position of 6.6 – 9.4 cM (POPSEQ) for both pairs (yellow circle). Green circle shows another difference for T46/N33 pair on 6H (telomere of the long arm).

7.4 Discussion

Forward genetics and map-based cloning approaches have been widely used for gene discovery in many plants. Lin et al. (2004) mapped a major QTL (*SKC1*) for shoot K^+ content in rice. The *SKC1* gene was isolated by map-based cloning and was revealed to encode a HKT-type transporter, known as *OsHKT1;5* (Ren et al. 2005). This is the first study which reveals a gene responsible for salt tolerance by forward genetics approach in monocot cereals (Takeda and Matsuoka 2008). In our study, fine mapping was conducted based on a major QTL for salinity tolerance which was detected before (Xu et al. 2012). Barley genomic sequences (http://www.ftpmips.helmholtz-muenchen.de/plants/barley/public_data/) facilitated the development of new molecular markers. Several polymorphic Indel markers

were developed, together with genotyping of DH population lines, to fine map this QTL. Flanking markers were determined through three recombinant lines (T83074, T83019 and T83045) of the DH population. These three lines are all tolerant to salinity stress, therefore target gene should be located in a 0.8 cM interval between Indel marker 19 and DArT marker 3268228d (Table 7.1). Morex contigs and genetic positions of these markers were also indicated according to the POPSEQ genetic map (Mascher et al. 2013). However, no recombinant line was found to be separated with markers (2086-5, 65, 103, 5257498d, 3268336d, 6792-1, 4783228d, 4782858d, 3259312d), which are closest to target gene at the moment. Bigger mapping populations are needed to find out more recombination. Even with all the new available technologies, fine mapping still relies on recombination between flanking markers. F_2 and NILs-derived populations will be further used to identify more recombinant lines.

Apart from fine mapping, candidate gene approach has also been used in many studies to find out the target gene. A *Nax2* locus responsible for Na^+ exclusion was identified in wheat, however, fine mapping of this gene was not possible due to a lack of recombination in the *Nax2* locus region (Munns et al. 2012). Candidate genes were identified through screening genes with similar phenotype for Na^+ exclusion in other species. An *OsHKT1;5*-like gene in rice was considered a candidate gene for Nax2 since it had a similar reduced rate of Na^+ transport from root to shoot (Munns et al. 2012; Ren et al. 2005). Another Na^+ exclusion locus (*HvNax3*) in was detected in barley (Shavrukov et al. 2010). Genes were predicted by comparative mapping to rice and *Brachypodium* in the *HvNax3* interval. *HVP10* (*LOC_Os06g08080*) encoding a vacuolar inorganic H^+-pyrophosphatase (V-ppase) was suggested the strong candidate gene (Shavrukov et al. 2013). In our study, blast searches with morex contigs of markers in our QTL region against rice genome were conducted. According to the high-resolution comparative analysis between barley and rice, barley 2H has high co-linearity with rice chromosome 4 and 7 (Mayer et al. 2011). However, only few markers (4783228d, 4782858d) were found to have homologous gene (*LOC_Os04g01520*, *LOC_Os04g01590*) in rice (data not shown).

NILs benefit from the fixed genetic background which avoids the noise from other genes or population structure, especially for quantitative traits (Chen et al. 2012a). NIL-derived populations allow the conversion of a quantitative trait to a Mendelian factor, which make it possible to fine mapping a QTL (Ma et al. 2012). NILs have been used for fine mapping in

many studies such as crown rot tolerance gene (Ma et al. 2012) and salinity tolerance gene (Munns et al. 2012) in wheat. Physiological characterization of two genes *Nax1* and *Nax2* using near isogenic homozygous lines indicated that both genes have lower rates of Na^+ and higher rates of K^+ transport from root to shoot (Huang et al. 2006). In current study, NILs were developed through marker assisted selection, and fast growing method using embryo culture. Indel marker 6792-1 was selected for screening heterozygotes for every generation until F_5. F_6 generation with different genotypes at 6792-1 came from F_5 self-breeding. Two pairs of near isogenic homozygous lines with similar genetic background (Figure 7.3) except the QTL region of our interest (6.6 - 9.4 cM) were selected, which exhibited different salinity tolerance (Figure 7.2). The pair of T46/N33 still showed a slight difference in growth rate during early seedling stage, which may due to the genotypic difference on chromosome 6H (Figure 7.3). These two pairs of near isogenic lines with different salinity tolerance have been further used for transcriptome analysis. The RNA-sequencing of NILs is in progress to detect the gene expression difference in transcriptional level, which could provide many candidate genes as references. Crosses will also be made between T66 and N53 to produce a large segregating population for the purpose of searching recombinant lines thus help further fine mapping. Meanwhile, physiological difference of these NILs will be analysed to investigate the possible mechanisms of this targeted locus/gene. These two pairs of NIL will also be tested in field for salinity tolerance, i.e. plant growth and grain yield.

In conclusion, better molecular markers were identified for one of the previously identified QTL for salinity tolerance on 2H. Using this marker, we delimitated a major QTL for salinity tolerance to 0.8 cM interval, and developed NILs through marker assisted selection which displayed different salinity tolerance. The pairs of NILs will provide excellent materials for further fine mapping and candidate gene searching for this QTL. This study also confirmed that Indel marker 6792-1 is an effective marker for selecting the salinity tolerance QTL on 2H.

Chapter 8 General conclusions and recommendations

8.1 Selection criterion for salinity tolerance

Even though genotyping has progressed rapidly, phenotyping remains a bottleneck because it is difficult to find reliable and convenient screening methods for salinity tolerance. In this study, several physiological traits have been evaluated for the possibilities of being used as selection criteria for salinity tolerance. These include ROS antioxidants, Na^+ content or Na^+/K^+ ratio, chlorophyll content, MDA and proline content in leaves. ROS antioxidant enzymes showed to be highly time- and tissue- dependent thus they cannot be used as biochemical indicators in breeding for salinity tolerance. As an indicator of lipid peroxidation, MDA content increased in all varieties under salinity stress conditions, therefore it is not suitable for using as screening criterion. Na^+ content or Na^+/K^+ ratio, proline and chlorophyll content were suggested as possible criteria for selecting salt tolerant varieties, due to higher chlorophyll content, lower Na^+/K^+ ratio and proline content discovered in tolerant varieties. In addition, stomatal and photosynthetic parameters were also proposed as potential selection criteria for plant salt tolerance in previous work by Liu et al. (2014). Na^+ content, proline content, stomatal and photosynthetic parameters were measured in different DH populations for QTL mapping afterwards, however, none of them was genetically linked to salinity tolerance. Therefore, although these physiological traits may be detected to be related with salinity tolerance at one particular snapshot or in some varieties, they could not represent the whole plant salinity tolerance.

8.2 QTL controlling salinity tolerance and related physiological or agronomical traits, and the linkage among them

In this study, two DH populations and one natural population with diverse germplasm were used for detecting QTL through bi-parental QTL mapping and genome wide association mapping respectively. In the natural population with 206 barley germplasm collections, a QTL on 4H for salinity tolerance was consistently detected in both GLM and MLM. This QTL located close to the telomere of 4HL. A QTL at a similar position controlling shoot Na^+/K^+ under saline conditions was previously reported by Long et al. (2013) but this QTL made no contribution to salinity tolerance. In the DH population from the cross of TX9425 and Franklin, one QTL for salinity tolerance was discovered on chromosome 7H, while one

for proline content on 3H and one for Na^+ content on 2H. This QTL on 7H for salinity tolerance was at a similar position to the QTL (*QST.YyFr.7H*) identified by Zhou et al. (2012) and another trait *HvNax3* on 7H controlling shoot Na^+ exclusion identified by Shavrukov et al. (2010). However, neither leaf Na^+ nor proline content showed correlation with salinity tolerance in this population. In another DH population from the cross of CM72 and Gairdner, one QTL for salinity tolerance was detected on 3H. When evaluating traits under stress condition, there were one QTL for stomata area on 1H, one QTL for leaf temperature on 2H, and two QTL for grain yield on 1H and 3H respectively. Only the QTL for grain yield on 3H was located at a similar position with the salinity tolerance QTL. Meanwhile, no common linkage was found between gas exchange and stomatal traits. Although gas exchange characteristics depend on stomatal form and structure, they are not necessarily genetically linked to each other. In addition, when evaluating under control condition, QTL for intercellular CO_2 concentration, transpiration rate and stomatal conductance were closely located together with that for biomass on chromosome 1H. Therefore, improving leaf photosynthetic capacity and genetic manipulation of photosynthesis are important approaches to enhance crop biomass (Horton 2000).

8.3 Future research recommendations

8.3.1 QTL validation and diagnostic markers developing for QTL pyramiding and MAS

In this study, a total of three QTL for salinity tolerance were detected locating on 3H, 4H and 7H, and lots of QTL were investigated for physiological or agronomical traits under salinity stress. The new QTL on 4H for salinity tolerance was detected by GWAS and this QTL should be further validated in bi-parental populations. For other QTL, especially the two QTL for salinity tolerance on 3H and 7H, diagnostic markers should be developed for them. The development of diagnostic markers will facilitate map-based gene cloning and promoting the efficiency for MAS in breeding salinity tolerant barley. The vital bases of successful breeding through MAS are the understanding of genetic traits and the selection of appropriate markers (Ashraf and Foolad 2013). The released physical, genetic and functional barley genome sequence assembly (Mayer et al. 2012) and the genetic map of Morex contigs from the POPSEQ (Mascher et al. 2013) can provide high density molecular markers which will facilitate new maker development. When diagnostic markers are developed which can

"represent" salinity tolerance, tolerant genes can be introgressed into elite variety through MAS and ultimate QTL pyramiding.

8.3.2 Map-based gene cloning and candidate genes investigation

The major QTL detected in the previous study has been fine mapped to a 0.8 cM interval in this study. The diagnostic markers developed in this study were used to develop near isogenic lines for further fine mapping. NILs based on the nearest marker derived from marker assisted selection exhibited contrast salinity tolerance, which proved the efficiency of MAS. The use of NIL can help to target the salinity gene more accurately and eliminate the noise caused by other genetic background, especially for quantitative traits (Chen et al. 2012a). Meanwhile, NIL-derived populations (crosses between two lines of a pair) allow the conversion of a quantitative trait to a Mendelian factor, which make it possible to fine map a QTL (Ma et al. 2012). Thus, NIL-derived population should be constructed to search for more recombinant lines for further high-throughput mapping and map-based cloning. In addition, RNA sequencing will be conducted to investigate candidate genes for this QTL using the NILs. Once candidate genes are found, they can be cloned for complementary test. Genes discovery will facilitate the in-depth understanding of salinity tolerance mechanisms, allow the utilization of gene specific markers for MAS, and even genome editing technologies for breeding salinity tolerant barley.

8.3.3 The effect of the tolerance loci on grain yield and other agronomic traits

The ultimate aim of the research is to transfer the outcome from lab and glasshouse to the field. NILs developed in this study will be evaluated for their salinity tolerance and grain yield in the field with a practical level of salinity. Only when the tolerance gene shows no negative effect on yield and other agronomic traits, it can be effectively introgressed into elite breeding lines through MAS.

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Appendix

Appendix 5.1 List of candidate genes within 10 cM around Bmac0209 on chromosome 3H

Gene Name	Chromosome	Phy_cM	Gene description
MLOC_6096.1	3	46.7	Beta-1,3-glucuronyltransferase, putative
MLOC_6097.1	3	46.7	Mitochondrial import inner membrane translocase subunit TIM14
MLOC_13599.2	3	46.9	Adenylate kinase
AK366836	3	46.9	alpha/beta-Hydrolases superfamily protein LENGTH=329
AK369041	3	46.9	ATP-dependent RNA helicase, putative
MLOC_19814.1	3	46.9	B-box zinc finger family protein
AK365312	3	46.9	Class III homeodomain-leucine zipper
MLOC_68042.1	3	46.9	Cysteine proteinase
MLOC_81291.1	3	46.9	Cytokinin oxidase/dehydrogenase
MLOC_16296.2	3	46.9	DNA-directed DNA polymerase
MLOC_62754.1	3	46.9	Endo-beta-1,4-glucanase
MLOC_59302.1	3	46.9	ERI1 exoribonuclease
AK252422.1	3	46.9	Gamma response I protein-like
AK361922	3	46.9	Gamma response I protein-like
MLOC_55828.2	3	46.9	Glucose-6-phosphate/phosphate translocator-related LENGTH=408
MLOC_67102.1	3	46.9	Myb family transcription factor
AK372831	3	46.9	O-fucosyltransferase family protein LENGTH=519
MLOC_31360.1	3	46.9	O-methyltransferase family protein, expressed
AK362624	3	46.9	Retinoblastoma binding protein, putative
AK361878	3	46.9	Serine/threonine-protein phosphatase 5
AK366918	3	46.9	Stachyose synthase
MLOC_59869.1	3	46.9	Thioredoxin-related transmembrane protein
MLOC_13598.3	3	46.9	Transposase
MLOC_42935.1	3	46.9	Unknown protein
MLOC_72568.3	3	46.9	Unknown protein
MLOC_4563.1	3	46.9	unknown protein
AK248260.1	3	46.9	Protein kinase (PK)
MLOC_12765.1	3	47.0	Calcium-dependent protein kinase
MLOC_54032.2	3	47.2	Phd finger protein
AK376641	3	47.4	TCP family transcription factor TCP4
MLOC_69218.1	3	47.5	Chitinase
MLOC_15787.2	3	47.5	RNA-dependent RNA polymerase family protein
MLOC_57556.1	3	47.5	Unknown protein
AK374529	3	47.8	Activating signal cointegrator 1 complex subunit
AK362702	3	47.8	Protein kinase-like protein
MLOC_61838.1	3	47.8	Zinc finger family protein
MLOC_16223.4	3	47.9	FBD-associated F-box protein LENGTH=459
MLOC_37050.1	3	47.9	Multidrug resistance protein ABC transporter family
MLOC_76564.1	3	48.1	ABC1 family protein

AK371666	3	48.1	Alpha/beta fold hydrolase family protein
AK370547	3	48.1	Bromodomain-containing factor
MLOC_19185.3	3	48.1	Isoamyl acetate-hydrolyzing esterase
MLOC_57121.2	3	48.1	Peroxidase 1
AK365832	3	48.1	Protein trichome birefringence-like 38
MLOC_69470.1	3	48.5	Basic helix-loop-helix (BHLH) family transcription factor
AK363627	3	48.5	Gamma-irradiation and mitomycin c induced 1
MLOC_77458.2	3	48.5	Poly(RC)-binding protein, putative
AK354947	3	48.5	Glycine-rich protein
MLOC_69640.2	3	48.6	Aquaporin 1
MLOC_211.2	3	48.6	ERI1 exoribonuclease
AK372187	3	48.6	Exportin-2
AK355166	3	48.6	Protein translocase subunit SecA
AK366049	3	48.6	Thioesterase family protein
MLOC_18337.2	3	48.7	GDSL esterase/lipase
MLOC_5530.2	3	48.7	S-receptor kinase, putative
AK366284	3	49.0	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme ThiJ
MLOC_64390.1	3	49.0	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
MLOC_51169.2	3	49.3	Alpha-1,2-mannosyltransferase ALG9
AK250517.1	3	49.3	ATP-dependent RNA helicase, putative
AK374758	3	49.3	BEST Arabidopsis thaliana protein match is: Late embryogenesis abundant protein
AK372570	3	49.3	BEST Arabidopsis thaliana protein match is: reduced male fertility
AK355163	3	49.3	Blue copper protein
MLOC_55325.1	3	49.3	Calcium-binding EF-hand family protein LENGTH=564
MLOC_9827.2	3	49.3	CBL-interacting protein kinase 5
MLOC_77266.1	3	49.3	CCR4-NOT transcription complex subunit
MLOC_13447.1	3	49.3	Chaperone protein dnaJ
AK359765	3	49.3	Cytochrome P450 family protein
MLOC_69476.1	3	49.3	Dual specificity protein phosphatase 9
MLOC_71849.2	3	49.3	Enoyl-CoA hydratase/isomerase family protein, expressed
MLOC_37813.1	3	49.3	ER glycerol-phosphate acyltransferase
MLOC_54188.1	3	49.3	F-box and associated interaction domains-containing protein LENGTH=417
MLOC_14078.1	3	49.3	Unknown protein
MLOC_53473.1	3	49.3	Glyoxylate/hydroxypyruvate reductase B
MLOC_5432.1	3	49.3	GTPase Der
MLOC_64351.2	3	49.3	Katanin p60 ATPase-containing subunit A-like protein
MLOC_9826.1	3	49.3	Kinase family protein
MLOC_8169.1	3	49.3	Metallothionein
MLOC_10556.1	3	49.3	Myb
MLOC_18081.1	3	49.3	nodulin-related protein 1 LENGTH=187
MLOC_54189.1	3	49.3	Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase A, putative
MLOC_63070.2	3	49.3	Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase A, putative
MLOC_74167.3	3	49.3	Phosphate translocator-like protein

MLOC_59333.1	3	49.3	Phosphoenolpyruvate carboxylase
MLOC_75157.4	3	49.3	Phospho-n-acetylmuramoyl-pentapeptide-transferase, putative
MLOC_16483.3	3	49.3	Protein of unknown function (DUF616) LENGTH=462
AK252472.1	3	49.3	Replication protein A 32 kDa subunit
AK374295	3	49.3	Replication protein A 32 kDa subunit
AK248270.1	3	49.3	Reticulon family protein
MLOC_3042.2	3	49.3	Retrotransposon protein, putative, Ty3-gypsy subclass
MLOC_5379.1	3	49.3	Ribosomal protein S6 family protein
MLOC_71051.1	3	49.3	Ring finger protein, putative
AK355156	3	49.3	Smr domain-containing protein
AK371590	3	49.3	SNF7 family protein
MLOC_18429.1	3	49.3	S-receptor kinase, putative
MLOC_6030.1	3	49.3	Stigma-specific Stig1 family protein
AK361337	3	49.3	tRNA (Guanine-N(1)-)-methyltransferase
AK359814	3	49.3	Uncharacterized conserved protein (DUF2358) LENGTH=241
AK374382	3	49.3	Unknown protein
MLOC_16481.1	3	49.3	Unknown protein
MLOC_62646.1	3	49.3	Unknown protein
MLOC_74954.1	3	49.3	Unknown protein
MLOC_81995.1	3	49.3	Unknown protein
AK371240	3	49.3	unknown protein
MLOC_60617.9	3	49.3	Unknown protein
MLOC_78172.1	3	49.3	Unknown protein
MLOC_37816.1	3	49.3	Unknown protein
AK252548.1	3	49.3	Unknown protein
MLOC_73581.1	3	49.3	Unknown protein
AK251615.1	3	49.3	Vesicle transport protein SFT2B, putative
MLOC_59332.2	3	49.3	Wiskott-Aldrich syndrome protein family member 2
AK250120.1	3	49.5	Unknown protein
MLOC_38385.1	3	49.6	Negatively light-regulated protein
MLOC_66539.5	3	49.6	Nodule inception protein
MLOC_55027.2	3	49.6	4-coumarate CoA ligase
MLOC_55025.1	3	49.6	Class E vacuolar protein-sorting machinery protein HSE1
AK363529	3	49.6	Cyclin A1
MLOC_37695.2	3	49.6	Heme-binding protein 2
MLOC_54586.1	3	50.4	S-receptor kinase-like
MLOC_634.2	3	50.4	Protein ABIL1
AK360111	3	50.5	ATP-binding protein of ABC transporter
AK368968	3	50.5	C2 domain-containing protein-like
AK373464	3	50.5	ERI1 exoribonuclease
MLOC_442.2	3	50.5	Expansin
AK373041	3	50.5	Flowering locus T
MLOC_36797.1	3	50.5	Kinesin-like protein
AK356763	3	50.5	Leucine-tRNA ligase
MLOC_5223.2	3	50.5	Lipase (Class 3)-like protein
MLOC_71066.1	3	50.5	myb domain protein 56 LENGTH=323

AK248551.1	3	50.5	NADH-cytochrome b5 reductase-like protein
MLOC_10261.1	3	50.5	Peroxidase 24
MLOC_12062.1	3	50.5	Protein kinase superfamily protein LENGTH=355
MLOC_38654.2	3	50.5	Retrotransposon protein, putative, LINE subclass
MLOC_75716.1	3	50.5	WPP domain-associated protein
MLOC_78652.1	3	50.5	Zinc finger family protein
AK360350	3	50.6	Glutaredoxin family protein
MLOC_81745.1	3	50.7	Respiratory burst oxidase-like protein B2
MLOC_63939.1	3	50.7	Pyruvate kinase
MLOC_63940.2	3	50.7	RING/U-box superfamily protein LENGTH=396
AK357938	3	50.7	ATP-dependent RNA helicase, putative
AK371869	3	50.8	Ankyrin repeat-containing protein, putative
MLOC_59292.1	3	50.8	Group 3 late embryogenesis abundant protein
AK366120	3	50.8	Lupus la ribonucleoprotein, putative
MLOC_59839.2	3	50.8	RING-H2 finger protein 2B LENGTH=147
MLOC_36305.2	3	51.0	cDNA, clone: J075123K08, full insert sequence
MLOC_56106.1	3	51.0	Phosphatidylinositol kinase family-like protein
AK358949	3	51.0	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme ThiJ
AK366717	3	51.1	ATP-dependent RNA helicase
MLOC_70538.1	3	51.1	Bromodomain-containing protein, putative
AK251942.1	3	51.1	FAD synthetase
MLOC_77422.3	3	51.1	Kinesin, putative
MLOC_45783.1	3	51.1	Nucleic acid-binding, OB-fold-like protein
MLOC_65275.1	3	51.1	Nup98 protein
MLOC_45782.1	3	51.1	Protein of unknown function (DUF1645) LENGTH=380
AK376395	3	51.1	Unknown protein
MLOC_57824.1	3	51.1	50S ribosomal protein L18
MLOC_10540.1	3	51.1	ATP-dependent RNA helicase, putative
AK248581.1	3	51.1	Beta-glucosidase, putative
MLOC_68387.1	3	51.1	flowering promoting factor 1 LENGTH=110
MLOC_57827.2	3	51.1	Pre-mRNA splicing factor prp46
MLOC_12866.1	3	51.1	Protein kinase-like protein
MLOC_54402.1	3	51.1	RING/U-box superfamily protein LENGTH=455
MLOC_68911.6	3	51.1	RING/U-box superfamily protein LENGTH=455
MLOC_12824.2	3	51.1	Unknown protein
MLOC_8252.1	3	51.2	Chaperone protein dnaJ 72
MLOC_15536.2	3	51.2	Kinase family protein
AK359887	3	51.2	Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase
AK366054	3	51.2	Alpha-glucosidase-like
MLOC_70493.1	3	51.2	BSD domain-containing protein
MLOC_77133.1	3	51.2	Cysteine proteinase inhibitor
MLOC_54047.1	3	51.2	Folate/biopterin transporter
MLOC_63492.1	3	51.2	HD domain containing protein
MLOC_2078.1	3	51.2	Hydroxymethylglutaryl-CoA lyase
AK248983.1	3	51.2	Importin alpha-1b subunit

AK248589.1	3	51.2	NEDD8-activating enzyme E1 catalytic subunit
AK369863	3	51.2	Pre-mRNA-splicing factor SPF27-like protein
MLOC_12117.1	3	51.2	Protein kinase superfamily protein LENGTH=361
AK354389	3	51.2	Protein kinase superfamily protein LENGTH=492
MLOC_70300.1	3	51.2	Protein of unknown function (DUF1218) LENGTH=257
AK252344.1	3	51.2	Protein of unknown function (DUF789) LENGTH=369
AK354110	3	51.2	Protein of unknown function DUF829, transmembrane 53
MLOC_58328.1	3	51.2	Protein of unknown function, DUF617 LENGTH=261
MLOC_10591.1	3	51.2	Rab gdp-dissociation inhibitor
MLOC_54049.1	3	51.2	Receptor-like kinase
MLOC_5202.2	3	51.2	Retrotransposon protein, putative, unclassified
MLOC_58975.3	3	51.2	Ribonuclease Z
AK363333	3	51.2	SAD1/UNC-84 domain protein 1 LENGTH=471
MLOC_7698.1	3	51.2	Transducin/WD40 repeat-like superfamily protein LENGTH=351
AK355669	3	51.2	Translin-associated protein X
AK353923	3	51.2	Triacylglycerol lipase
AK252435.1	3	51.2	Unknown protein
MLOC_65945.1	3	51.2	Auxin response factor
MLOC_61990.1	3	51.3	Expansin A2
MLOC_13163.1	3	51.3	Unknown protein
AK360949	3	51.3	ADP-ribosylation factor family protein
MLOC_56598.1	3	51.3	Dof zinc finger protein
AK375738	3	51.3	GDSL esterase/lipase
MLOC_13377.3	3	51.3	glucan synthase-like 7 LENGTH=1958
MLOC_501.1	3	51.3	glucan synthase-like 7 LENGTH=1958
AK366122	3	51.3	Isopenicillin N epimerase
MLOC_59050.1	3	51.3	Malate dehydrogenase
MLOC_56595.1	3	51.3	mRNA, clone: RTFL01-16-D19
MLOC_56597.2	3	51.3	Pentatricopeptide repeat-containing protein
MLOC_56594.1	3	51.3	Peroxidase 72
MLOC_74879.2	3	51.3	Potassium channel
MLOC_71295.1	3	51.3	WD-40 repeat protein
AK363508	3	51.3	Protein of unknown function (DUF630 and DUF632)
AK370533	3	51.3	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase
MLOC_70839.3	3	51.3	6-phosphogluconate dehydrogenase, NAD-binding protein
MLOC_75225.1	3	51.3	Aldo/keto reductase, putative
MLOC_10478.2	3	51.3	ATP dependent RNA helicase, putative
AK249478.1	3	51.3	ATP-dependent Clp protease proteolytic subunit
MLOC_56183.1	3	51.3	calmodulin 3 LENGTH=149
MLOC_5725.1	3	51.3	Carbamoyl-phosphate synthase large chain
MLOC_11651.1	3	51.3	Carbonic anhydrase
MLOC_43331.1	3	51.3	Carbonic anhydrase
MLOC_68238.3	3	51.3	Casein kinase I
MLOC_7481.2	3	51.3	CRAL-TRIO domain-containing protein
AK361038	3	51.3	DET1-like protein
AK249489.1	3	51.3	Developmentally-regulated GTP-binding protein 2

AK250646.1	3	51.3	DNA-directed RNA polymerase subunit beta
MLOC_79421.2	3	51.3	Dof zinc finger protein
MLOC_15661.3	3	51.3	Elongation factor
AK358149	3	51.3	GDSL esterase/lipase
AK357129	3	51.3	Glucosidase II beta subunit, putative
AK249939.1	3	51.3	Glutamate dehydrogenase, putative
AK369992	3	51.3	Glutamate dehydrogenase, putative
MLOC_4846.1	3	51.3	histone-lysine N-methyltransferases LENGTH=265
MLOC_17789.4	3	51.3	Holocarboxylase synthetase
MLOC_71648.1	3	51.3	Leucine-rich repeat receptor-like protein kinase family protein
AK354074	3	51.3	Myosin XI, putative
MLOC_11983.1	3	51.3	Nuclear-interacting partner of ALK
MLOC_71177.2	3	51.3	Pectinesterase
MLOC_79423.3	3	51.3	Phospholipid-transporting atpase, putative
MLOC_4738.2	3	51.3	Polygalacturonase
AK354548	3	51.3	'Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 '
MLOC_72694.1	3	51.3	Protein FAM91A1
MLOC_60329.4	3	51.3	Protein of Unknown Function (DUF239) LENGTH=465
MLOC_60676.1	3	51.3	Protein SEY1
MLOC_55497.1	3	51.3	Protein transport protein SEC23
AK252927.1	3	51.3	Protein transport protein Sec61 beta subunit
AK251523.1	3	51.3	Protein trichome birefringence-like 19
AK249076.1	3	51.3	Ras-related protein Rab-1A
MLOC_15683.1	3	51.3	Rwd domain-containing protein, putative
AK248727.1	3	51.3	Serine/threonine protein phosphatase 6 regulatory subunit
AK369840	3	51.3	Serine/threonine-protein phosphatase
MLOC_52841.1	3	51.3	Tudor/PWWP/MBT superfamily protein LENGTH=645
MLOC_20220.1	3	51.3	Unknown protein
AK371824	3	51.3	Unknown protein
MLOC_31807.1	3	51.3	Unknown protein
MLOC_17622.1	3	51.3	Unknown protein
MLOC_21220.1	3	51.3	Uroporphyrinogen decarboxylase
MLOC_13658.1	3	51.3	Vacuolar cation/proton exchanger 1b
MLOC_63030.1	3	51.3	V-type proton ATPase subunit F
MLOC_5666.3	3	51.3	Zinc finger C-x8-C-x5-C-x3-H type family protein LENGTH=310
MLOC_76295.4	3	51.4	Kelch domain-containing protein
MLOC_34067.1	3	51.4	Pentatricopeptide repeat-containing protein
MLOC_21279.1	3	51.4	Phosphoribosyl-AMP cyclohydrolase
MLOC_28262.1	3	51.4	Unknown protein
AK372868	3	51.4	F-box family protein
MLOC_76747.1	3	51.4	Histone H2B
MLOC_70040.1	3	51.4	Inositol-1-monophosphatase
AK361084	3	51.4	Lipase
AK249459.1	3	51.4	Major facilitator superfamily antiporter
MLOC_70039.3	3	51.4	NAD dependent epimerase/dehydratase, putative
MLOC_316.1	3	51.4	Retrotransposon protein, putative, unclassified

MLOC_62294.1	3	51.4	Retrotransposon protein, putative, unclassified
AK376113	3	51.4	Similarity to kinesin protein
MLOC_61398.2	3	51.4	Small nuclear ribonucleoprotein SM D3, putative
MLOC_60240.4	3	51.4	TRAM, LAG1 and CLN8 (TLC) lipid-sensing domain containing protein LENGTH=231
MLOC_62295.1	3	51.4	Unknown protein
AK367613	3	51.5	Protein kinase, putative
MLOC_62922.1	3	51.5	50S ribosomal protein L25
MLOC_73950.1	3	51.5	Amino acid permease
AK357181	3	51.5	BEST Arabidopsis thaliana protein match is: F-box family proteinLENGTH=279
MLOC_54627.2	3	51.5	Coatomer subunit beta
AK371962	3	51.5	JmjC domain-containing protein
MLOC_62245.3	3	51.5	Kinase family protein
MLOC_10575.1	3	51.5	Peptidase M16 family protein
MLOC_11645.3	3	51.5	Peptidase M16 family protein
AK370389	3	51.5	Peptide deformylase
MLOC_67396.4	3	51.5	Protein of unknown function (DUF3531) LENGTH=302
MLOC_62921.1	3	51.5	Protein of unknown function (DUF581) LENGTH=344
MLOC_4508.2	3	51.5	Protein SYS1
MLOC_11647.1	3	51.5	Retrotransposon protein, putative, unclassified
MLOC_79155.1	3	51.5	Serine/threonine protein phosphatase 2A 55 kDa regulatory subunit B beta isoform
AK376175	3	51.5	Ubiquitin-like superfamily protein LENGTH=215
MLOC_61662.2	3	51.5	Unknown protein
MLOC_15864.2	3	51.5	WD repeat protein
AK248922.1	3	51.5	Zinc finger CCCH domain-containing protein 7
MLOC_52106.6	3	51.5	Zinc finger CCCH domain-containing protein 7
AK251631.1	3	51.5	Zinc finger CCCH domain-containing protein 8
MLOC_10981.1	3	51.6	Cysteine proteinase-like
MLOC_66447.1	3	51.6	Pectinesterase
MLOC_14196.1	3	51.6	Unknown protein
MLOC_60912.1	3	51.6	Unknown protein
AK360890	3	51.6	Replication protein A 70 kDa DNA-binding subunit
AK367337	3	51.6	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
AK361192	3	51.6	30S ribosomal protein S1-like
AK364981	3	51.6	3'-5' exonuclease domain-containing protein-like
AK366955	3	51.6	5-nucleotidase
MLOC_64458.1	3	51.6	Acyl transferase
MLOC_63091.5	3	51.6	Acyl-protein thioesterase 2
MLOC_32718.4	3	51.6	Adaptin ear-binding coat-associated protein 1
AK362601	3	51.6	Amino acid transporter, putative
MLOC_54580.10	3	51.6	Argonaute 4-like protein
MLOC_68778.1	3	51.6	ATPase, putative
AK371692	3	51.6	ATP-binding cassette transporter, putative
MLOC_56149.1	3	51.6	ATP-dependent RNA helicase
MLOC_56244.1	3	51.6	ATP-dependent RNA helicase

AK250178.1	3	51.6	ATP-dependent zinc metalloprotease FtsH
AK356297	3	51.6	ATP-dependent zinc metalloprotease FtsH 1
MLOC_59013.3	3	51.6	ATP-dependent zinc metalloprotease FtsH 2
MLOC_34621.1	3	51.6	Beta-galactosidase
MLOC_11568.1	3	51.6	BY-2 kinesin-like protein 10
AK357239	3	51.6	BZIP transcription factor
AK359391	3	51.6	BZIP transcription factor
AK361113	3	51.6	C4-dicarboxylate transporter/malic acid transport protein
AK248914.1	3	51.6	Casein kinase I-like
MLOC_63506.1	3	51.6	Cell growth-regulating nucleolar protein, putative
AK363628	3	51.6	Chaperone protein dnaJ
MLOC_68007.2	3	51.6	Chloroplast unusual positioning 1A
AK249432.1	3	51.6	Choline dehydrogenase
AK363968	3	51.6	CHY zinc finger containing protein
MLOC_7183.1	3	51.6	Coatomer subunit beta
AK353646	3	51.6	Coiled-coil domain-containing protein, putative
AK367974	3	51.6	Condensin complex subunit 2
AK369234	3	51.6	CTP synthase
MLOC_44702.1	3	51.6	Cytochrome P450-like
AK250307.1	3	51.6	Dehydroquinate dehydratase/ shikimate dehydrogenase
MLOC_72321.2	3	51.6	DNA (Cytosine-5)-methyltransferase, putative
MLOC_15623.1	3	51.6	DNA binding protein
MLOC_64457.1	3	51.6	DNA repair and recombination protein RAD54-like protein
MLOC_61674.1	3	51.6	DUF866-domain-containing protein
MLOC_20645.2	3	51.6	Esterase/lipase/thioesterase family protein-like
MLOC_19320.2	3	51.6	Eukaryotic aspartyl protease family protein LENGTH=485
MLOC_53255.7	3	51.6	Eukaryotic translation initiation factor 3 subunit 10-like
MLOC_58301.1	3	51.6	Exosome complex exonuclease RRP40
MLOC_74504.1	3	51.6	F-box family protein
MLOC_82043.1	3	51.6	Gag-pol polyprotein
MLOC_54200.2	3	51.6	GDSL esterase/lipase
MLOC_67987.1	3	51.6	Glucose-repressible alcohol dehydrogenase transcriptional effector
MLOC_61627.1	3	51.6	Glutamine-tRNA ligase
MLOC_72705.2	3	51.6	Glycosyl transferase family 1 protein
MLOC_6029.2	3	51.6	GPI transamidase component gaa1
MLOC_53680.1	3	51.6	HAT dimerisation domain-containing protein-like
MLOC_21667.1	3	51.6	HAT family dimerization domain-containing protein
MLOC_11453.1	3	51.6	Heat shock transcription factor
MLOC_13110.1	3	51.6	Heavy metal transport/detoxification superfamily protein
AK365152	3	51.6	Histone H1.1, putative
MLOC_16644.1	3	51.6	HSP20-like chaperones superfamily protein LENGTH=249
AK361432	3	51.6	IAA-amino acid hydrolase ILR1, putative
MLOC_51868.2	3	51.6	Kinase family protein
MLOC_55928.2	3	51.6	Kinase family protein
MLOC_59055.2	3	51.6	Kinase, putative
MLOC_58522.2	3	51.6	Kinesin-like protein

AK368412	3	51.6	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AK370515	3	51.6	Late embryogenesis abundant hydroxyproline-rich glycoprotein
MLOC_72263.2	3	51.6	Late-embryogenesis-abundant protein
MLOC_59173.1	3	51.6	Lipase class 3-like
AK364616	3	51.6	Lipase family protein, expressed
AK363519	3	51.6	LisH domain and HEAT repeat-containing protein KIAA1468 homolog
MLOC_14393.3	3	51.6	Loricrin-like
MLOC_51282.1	3	51.6	MBOAT (membrane bound O-acyl transferase) family protein
MLOC_52550.1	3	51.6	Meiosis 5
MLOC_12284.1	3	51.6	Mitochondrial ATP synthase g subunit family protein
MLOC_4592.2	3	51.6	Mitochondrial carrier protein-like
MLOC_61626.3	3	51.6	Mitochondrial import inner membrane translocase subunit Tim17/22/23 family protein
MLOC_7981.1	3	51.6	MYB-related transcription factor
MLOC_52460.1	3	51.6	Neutral ceramidase
MLOC_62684.1	3	51.6	NHL domain-containing protein LENGTH=387
MLOC_55760.4	3	51.6	NMDA receptor-regulated protein, putative
MLOC_38362.2	3	51.6	OHP2
AK368921	3	51.6	Oxidoreductase
MLOC_16721.1	3	51.6	Pectin lyase-like superfamily protein LENGTH=404
MLOC_36440.1	3	51.6	Pectin lyase-like superfamily protein LENGTH=444
MLOC_38363.1	3	51.6	Pentatricopeptide repeat-containing protein
MLOC_58033.1	3	51.6	Pentatricopeptide repeat-containing protein
MLOC_6067.1	3	51.6	Pentatricopeptide repeat-containing protein
MLOC_52822.2	3	51.6	Pentatricopeptide repeat-containing protein, putative
MLOC_59847.1	3	51.6	Pentatricopeptide repeat-containing protein, putative
MLOC_69201.1	3	51.6	Pentatricopeptide repeat-containing protein, putative
AK249079.1	3	51.6	Peroxidase
MLOC_11639.4	3	51.6	PHD-finger family protein
AK252135.1	3	51.6	Phenazine biosynthesis protein PhzF family
MLOC_16151.2	3	51.6	Phenazine biosynthesis protein PhzF family
AK248230.1	3	51.6	Polypyrimidine tract-binding protein 1-like
MLOC_16643.2	3	51.6	Porin/voltage-dependent anion-selective channel protein
AK361243	3	51.6	PQ loop repeat family protein
MLOC_52548.1	3	51.6	Proteasome-associated protein ECM29-like protein
MLOC_60540.2	3	51.6	Protein ABIL1
AK359804	3	51.6	Protein kinase family protein
MLOC_66658.1	3	51.6	Protein kinase family protein with ARM repeat domain
AK367636	3	51.6	Protein kinase superfamily protein LENGTH=400
AK251292.1	3	51.6	Protein kinase superfamily protein LENGTH=493
MLOC_5991.1	3	51.6	Protein kinase superfamily protein LENGTH=493
AK251287.1	3	51.6	Protein kinase, putative
MLOC_66657.2	3	51.6	Protein kinase, putative
AK249272.1	3	51.6	Protein midA-like protein, mitochondrial
MLOC_72316.1	3	51.6	Protein of unknown function (DUF1118) LENGTH=198

AK365128	3	51.6	Protein of unknown function (DUF1230) LENGTH=327
MLOC_34502.1	3	51.6	Protein of unknown function (DUF1644) LENGTH=334
AK363708	3	51.6	Protein of unknown function (DUF3133) LENGTH=768
MLOC_60762.1	3	51.6	Protein of unknown function (DUF630 and DUF632)
AK248235.1	3	51.6	Protein of unknown function (DUF803) LENGTH=344
AK358947	3	51.6	Protein of unknown function, DUF538 LENGTH=151
AK251854.1	3	51.6	Protein phosphatase 2C
MLOC_52823.1	3	51.6	Pyridoxal phosphate phosphatase PHOSPHO2, putative
MLOC_15249.5	3	51.6	Regulator of telomere elongation helicase 1
MLOC_63257.1	3	51.6	Retrotransposon protein, putative, Ty1-copia subclass
MLOC_76359.1	3	51.6	Retrotransposon protein, putative, Ty1-copia subclass
MLOC_23302.1	3	51.6	Retrotransposon protein, putative, unclassified
MLOC_38170.1	3	51.6	Retrotransposon protein, putative, unclassified
MLOC_55762.2	3	51.6	Retrotransposon protein, putative, unclassified
AK374126	3	51.6	Ribosomal RNA apurinic site specific lyase
MLOC_14403.1	3	51.6	RNA-directed DNA polymerase (Reverse transcriptase)
MLOC_12285.1	3	51.6	Sad1/UNC domain protein
MLOC_56249.1	3	51.6	SEC22 vesicle trafficking protein-like protein B
MLOC_55189.4	3	51.6	Serine/threonine protein phosphatase 4 regulatory subunit
AK371948	3	51.6	SGT1
MLOC_72378.1	3	51.6	Small subunit processome component-like protein
AK368425	3	51.6	Subtilase
MLOC_53418.2	3	51.6	Tetratricopeptide repeat protein 2-like
AK358001	3	51.6	Transcriptional activator protein Pur-alpha
MLOC_67947.1	3	51.6	Transposon protein, putative, CACTA, En/Spm sub-class, expressed
MLOC_72054.2	3	51.6	Triacylglycerol lipase, putative
AK363729	3	51.6	Ubiquitin carboxyl-terminal hydrolase
MLOC_56933.3	3	51.6	Ubiquitin-conjugating enzyme X
MLOC_20377.3	3	51.6	Ubiquitin-like modifier-activating enzyme ATG7
AK353750	3	51.6	Unknown protein
MLOC_5065.3	3	51.6	Unknown protein
MLOC_62290.1	3	51.6	Unknown protein
MLOC_6682.1	3	51.6	Unknown protein
MLOC_67968.1	3	51.6	Unknown protein
AK249929.1	3	51.6	Unknown protein
MLOC_59875.1	3	51.6	Unknown protein
MLOC_54626.1	3	51.6	Unknown protein
AK376084	3	51.6	Unknown protein
MLOC_16828.4	3	51.6	Unknown protein
MLOC_74528.3	3	51.6	Unknown protein
MLOC_67604.1	3	51.6	Unknown protein
MLOC_52825.1	3	51.6	Unknown protein
MLOC_52821.2	3	51.6	Unknown protein
MLOC_52549.1	3	51.6	Unknown protein
MLOC_11491.1	3	51.6	Unknown protein
MLOC_4196.1	3	51.6	Unknown protein

MLOC_51217.1	3	51.6	Unknown protein
AK361526	3	51.6	UPF0052 domain protein
MLOC_59407.1	3	51.6	Vacuolar processing enzyme 3
AK251977.1	3	51.6	V-type proton ATPase subunit d2
AK370701	3	51.6	WD-40 repeat protein
AK375065	3	51.6	WD-repeat protein, putative
AK377138	3	51.6	WD-repeat protein, putative
MLOC_67697.1	3	51.6	WD-repeat protein, putative
AK368513	3	51.6	WRKY transcription factor 12
MLOC_54895.1	3	51.6	WRKY transcription factor, putative
AK363414	3	51.6	XH/XS domain-containing protein LENGTH=634
AK374335	3	51.6	XH/XS domain-containing protein LENGTH=634
MLOC_11172.2	3	51.6	Yip1 domain-containing protein
MLOC_71854.1	3	51.6	Yth domain-containing protein, putative
MLOC_52138.6	3	51.6	Zinc finger CCCH domain-containing protein 3
AK371626	3	51.6	Zinc finger CCCH domain-containing protein 6
MLOC_16487.1	3	51.6	Zinc finger protein LSD1
AK362855	3	51.6	Zinc finger protein-like protein
MLOC_15120.1	3	51.6	Zinc finger-like
MLOC_4591.1	3	51.6	ZmGR2c protein
MLOC_5786.2	3	51.7	Auxilin-like protein
MLOC_6128.3	3	51.7	Auxin efflux carrier
MLOC_65788.2	3	51.7	Dihydroflavonol-4-reductase
MLOC_63284.1	3	51.7	Glucan endo-1,3-beta-glucosidase-like protein
MLOC_56354.3	3	51.7	Protein kinase family protein
MLOC_75655.4	3	51.7	SNF1-related protein kinase regulatory subunit gamma
MLOC_16375.1	3	51.7	Unknown protein
MLOC_54754.1	3	51.8	50S ribosomal protein L31
MLOC_52238.1	3	51.8	Adenylate isopentenyltransferase
AK371372	3	51.8	Cinnamoyl CoA reductase-like
AK353842	3	51.8	ER glycerol-phosphate acyltransferase
MLOC_55339.1	3	51.8	homeobox-leucine zipper protein 17 LENGTH=275
MLOC_7247.1	3	51.8	Kinase, putative
MLOC_5568.1	3	51.8	myb domain protein 21 LENGTH=226
AK366572	3	51.8	PITH domain containing 1
MLOC_56451.1	3	51.8	Siroheme synthase
AK357374	3	51.8	Ulp1 protease family, C-terminal catalytic domain containing protein, expressed
AK354291	3	52.0	OBP3-responsive gene 1 LENGTH=670
MLOC_58312.1	3	52.1	30S ribosomal protein S20
MLOC_10854.2	3	52.3	Sodium Bile acid symporter family LENGTH=409
MLOC_13604.1	3	52.4	Glutamate synthase, putative
MLOC_17211.14	3	52.4	DNA ligase
MLOC_19634.2	3	52.4	DNA ligase
MLOC_15556.1	3	52.4	MATE efflux protein-like
MLOC_4609.1	3	52.4	Mitogen activated protein kinase 20-4

AK361328	3	52.4	Ribokinase
MLOC_14545.2	3	52.4	UDP-Glycosyltransferase superfamily protein LENGTH=479
AK366123	3	52.6	ATP synthase subunit beta
MLOC_69600.1	3	52.6	Isocitrate dehydrogenase [NADP]
MLOC_12156.1	3	52.6	Microtubule-associated protein-like
AK374059	3	52.6	Protein phosphatase 2c, putative
MLOC_548.2	3	52.6	Ras-related protein Rab-25
AK248203.1	3	52.6	Regulator of Vps4 activity in the MVB pathway protein
AK356259	3	52.6	RING-finger protein-like
AK356876	3	52.6	Splicing factor 3A subunit 3, putative, expressed
AK358857	3	52.6	Transcription initiation factor TFIID subunit 11
MLOC_7925.1	3	52.6	WUSCHEL-related homeobox
AK367767	3	52.8	GDSL esterase/lipase
MLOC_56501.2	3	52.8	Myosin heavy chain-like
AK360231	3	52.8	Protein kinase, putative
MLOC_44455.1	3	52.8	WRKY transcription factor 21
MLOC_55781.5	3	52.9	D-glycerate 3-kinase
MLOC_62571.1	3	52.9	Homeobox protein
MLOC_64487.1	3	52.9	Homeobox protein, putative
MLOC_65417.5	3	52.9	Protein DEHYDRATION-INDUCED 19 homolog 3
MLOC_78236.1	3	52.9	Protein of unknown function (DUF1421) LENGTH=496
MLOC_78237.1	3	52.9	Sigma DNA polymerase, putative
MLOC_15173.1	3	52.9	Ubiquitin-conjugating enzyme E2 N
MLOC_12954.1	3	53.1	Peroxiredoxin-5
MLOC_56360.3	3	53.1	Protein kinase, putative
MLOC_51570.1	3	53.1	Receptor protein kinase, putative
MLOC_12953.1	3	53.1	Rop guanine nucleotide exchange factor
MLOC_64975.2	3	53.1	TUBBY protein
MLOC_6125.10	3	53.1	Arginine/serine-rich splicing factor, putative
MLOC_5049.1	3	53.3	26S protease regulatory subunit 4 homolog
MLOC_56911.1	3	53.3	60S ribosomal protein L37a
AK365950	3	53.3	Abhydrolase domain-containing protein FAM108C1
MLOC_57391.1	3	53.3	Acidic endochitinase
MLOC_11735.1	3	53.3	ARP2/3 complex 34 kDa subunit
AK355233	3	53.3	ATP synthase protein I
AK359596	3	53.3	ATP synthase protein I
AK374212	3	53.3	Carboxyl-terminal-processing protease
MLOC_64727.3	3	53.3	Clathrin assembly protein
MLOC_11734.3	3	53.3	CTP synthase
MLOC_4453.1	3	53.3	Cytochrome b561/ferric reductase transmembrane family protein
MLOC_5835.2	3	53.3	Cytochrome b561/ferric reductase transmembrane family protein
MLOC_43543.1	3	53.3	F-box domain containing protein
MLOC_65158.1	3	53.3	F-box-like protein
AK356294	3	53.3	GPN-loop GTPase
MLOC_7069.1	3	53.3	High mobility group family protein
AK374057	3	53.3	Hydroxyacylglutathione hydrolase

MLOC_140.5	3	53.3	Kinase R-like protein
MLOC_64728.1	3	53.3	Kinase-like protein
AK248363.1	3	53.3	Kinase-related protein of unknown function (DUF1296)
MLOC_11738.2	3	53.3	Membrane-bound transcription factor site-2 protease
AK366322	3	53.3	N-methyltransferase 1
MLOC_55132.2	3	53.3	Nucleotidylyl transferase superfamily protein LENGTH=388
AK356012	3	53.3	Peroxidase 19, putative
MLOC_11190.3	3	53.3	Protein kinase family protein
AK360556	3	53.3	Protein kinase superfamily protein LENGTH=654
AK372355	3	53.3	Protein kinase superfamily protein LENGTH=654
MLOC_62988.1	3	53.3	Protein of unknown function (DUF1639) LENGTH=179
MLOC_57393.1	3	53.3	Pyruvate kinase
AK365590	3	53.3	Rad25/xp-B DNA repair helicase, putative
MLOC_55131.3	3	53.3	Reticulon family protein LENGTH=225
MLOC_36229.1	3	53.3	Retrotransposon protein, putative, unclassified
MLOC_69110.4	3	53.3	RING/U-box superfamily protein LENGTH=634
AK357370	3	53.3	RNA polymerase III subunit-like protein
MLOC_12312.1	3	53.3	RNA polymerase III subunit-like protein
AK250359.1	3	53.3	Serine/threonine-protein phosphatase
MLOC_1317.1	3	53.3	S-receptor kinase-like
AK364738	3	53.3	Transducin/WD40 repeat-like superfamily protein LENGTH=1377
AK248968.1	3	53.3	Ubiquitin conjugating enzyme E2
MLOC_66069.1	3	53.3	Unknown protein
MLOC_44301.1	3	53.3	Unknown protein
AK363511	3	53.3	UPF0678 fatty acid-binding protein-like protein
MLOC_57866.1	3	53.3	V-type proton ATPase subunit E1
AK362560	3	53.6	CASC3/Barentsz eIF4AIII binding LENGTH=605
MLOC_62048.1	3	53.6	CASC3/Barentsz eIF4AIII binding LENGTH=605
MLOC_56928.1	3	53.9	3-beta-glucuronosyltransferase
AK371339	3	53.9	Eukaryotic aspartyl protease family protein LENGTH=461
AK369681	3	54.2	AP2 domain-containing transcription factor
AK358661	3	54.2	Chitinase
MLOC_62570.3	3	54.2	Peptidyl-tRNA hydrolase
MLOC_59852.2	3	54.2	Symplekin, putative
MLOC_57388.1	3	54.2	Threonine synthase-like
AK356673	3	54.5	Unknown protein
AK372847	3	54.5	CASP-like protein
MLOC_68295.1	3	54.5	Glutathione-S-transferase 2
MLOC_7947.1	3	54.5	Lipid phosphate phosphatase 3
AK353628	3	54.6	Protein phosphatase, putative
AK354505	3	54.8	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein
MLOC_54191.1	3	54.8	ERI1 exoribonuclease
MLOC_60806.5	3	55.1	ABC transporter ATP-binding protein
AK363190	3	55.1	Lipid A export ATP-binding/permease protein MsbA
MLOC_18521.1	3	55.1	Outward rectifying potassium channel

MLOC_18520.5	3	55.1	Unknown protein
MLOC_60474.3	3	55.2	Calcineurin B-like protein 06
MLOC_73985.1	3	55.2	HAT family dimerization domain-containing protein
MLOC_3643.1	3	55.2	Protein kinase-like
AK366559	3	55.2	SWAP (Suppressor-of-White-APricot)/surp domain-containing protein LENGTH=844
MLOC_73984.1	3	55.2	Unknown protein
MLOC_67147.1	3	55.2	VIP1 protein
MLOC_70215.1	3	55.7	F-box domain containing protein, expressed
AK368147	3	55.7	GH3
MLOC_18177.4	3	55.7	Myb domain protein 26
AK360453	3	55.7	Myosin heavy chain-like
MLOC_37776.4	3	55.7	AP-1 complex subunit mu
MLOC_63996.1	3	55.7	Bidirectional sugar transporter N3
AK353559	3	55.7	Farnesyl pyrophosphate synthase
MLOC_60990.1	3	55.7	GDSL esterase/lipase
AK354325	3	55.7	Kelch repeat-containing F-box-like protein
MLOC_75087.1	3	55.7	Nitrate transporter
AK365977	3	55.7	Plastid transcriptionally active7
MLOC_4116.1	3	55.7	S-adenosyl-L-methionine:carboxyl methyltransferase family protein
MLOC_7040.1	3	55.7	Subtilisin-like serine protease
MLOC_34719.3	3	55.7	Transducin/WD40 domain-containing protein
MLOC_38922.1	3	55.7	Unknown protein
MLOC_63995.1	3	55.7	Unknown protein
AK367282	3	55.7	Unknown protein
AK370652	3	55.8	IAA-amino acid hydrolase ILR1-like 4
MLOC_4753.2	3	55.8	Late embryogenesis abundant protein
AK362485	3	55.8	MAPK activating protein-like
AK369626	3	55.8	RNA polymerase II transcriptional coactivator
MLOC_7612.1	3	55.8	Vesicle transport v-SNARE 13
MLOC_70409.1	3	56.2	Basic helix-loop-helix (BHLH) family transcription factor
AK355455	3	56.2	BHLH transcription factor-like
MLOC_2059.2	3	56.2	Chaperone protein dnaJ 10
AK372534	3	56.2	myb domain protein 86 LENGTH=352
AK375619	3	56.2	Single myb histone 4
MLOC_63875.1	3	56.4	Protein transport protein Sec61 subunit alpha
MLOC_19332.3	3	56.4	Sec14p-like phosphatidylinositol transfer family protein
MLOC_57123.4	3	56.4	Voltage-gated chloride channel, putative
AK248991.1	3	56.6	AP-2 complex subunit beta-1, putative
MLOC_11565.1	3	56.6	Mitochondrial import receptor subunit TOM7-1
MLOC_55669.1	3	56.7	IQ domain-containing protein
MLOC_78658.1	3	56.7	Mitochondrial carrier protein-like

Appendix 6.1 The value of population structure of 206 accessions. Each accession belongs to the population with the highest value calculated by STRUCTURE software

Trait	Q1	Q2	Q3	Q4	Q5	Q6
Russia6	0.001	0.997	0	0.001	0.001	0
WA12916	0	0.997	0	0.001	0.001	0
YHZWB	0.001	0.997	0.001	0.001	0.001	0
CI-8826	0	0.997	0	0.001	0.001	0
Svanhals	0	0.997	0	0.001	0.001	0
KyotoNakate	0.001	0.637	0.002	0.01	0.348	0.001
Harbin-2-Ro	0.001	0.714	0.263	0.002	0.02	0
Svansota	0.003	0.618	0.261	0.023	0.094	0.001
JSELM	0.001	0.674	0.014	0.002	0.002	0.307
WA12915	0.001	0.822	0.001	0.001	0.001	0.175
WA12927	0.001	0.591	0.001	0.001	0.001	0.406
WA12923	0.004	0.885	0.001	0.007	0.002	0.101
WA12924	0.002	0.8	0.001	0.003	0.011	0.183
TF026	0.019	0.575	0.002	0.063	0.323	0.018
TX9425	0.012	0.878	0.001	0.094	0.002	0.013
ZUG403	0.023	0.686	0.001	0.18	0.094	0.015
93-3143	0.008	0.768	0.03	0.002	0.001	0.19
B1043	0.001	0.756	0.125	0.001	0.001	0.117
WA12910	0.001	0.774	0.001	0.021	0.09	0.113
WA12935	0.001	0.836	0.001	0.046	0.012	0.104
WA12899	0.001	0.866	0	0.001	0.001	0.131
WA12936	0.001	0.861	0.001	0.001	0.001	0.135
B1052	0.001	0.917	0.001	0.001	0.078	0.002
WA12925	0.001	0.938	0.001	0.004	0.004	0.052
WA12906	0.001	0.837	0.001	0.001	0.001	0.161
WA12908	0.01	0.638	0.009	0.126	0.001	0.216
B1064	0.001	0.997	0.001	0.001	0.001	0
WA12900	0	0.997	0	0.001	0.001	0
WA12928	0.041	0.933	0.022	0.001	0.002	0.001
WA12903	0.001	0.994	0.001	0.002	0.001	0.001
WA12905	0.001	0.994	0.001	0.001	0.003	0.001
YU6472	0.009	0.941	0.001	0.013	0.013	0.022
WA12902	0.001	0.833	0.134	0.003	0.017	0.011
B1133	0.001	0.822	0.001	0.001	0.003	0.172
YWHKSL	0.001	0	0.001	0.001	0.001	0.998
WA12896	0.005	0.733	0.009	0.001	0.006	0.246
Aizao3	0.114	0.534	0.022	0.207	0.106	0.017
Lixi143	0.001	0.79	0.12	0.082	0.004	0.002
Nasonijo	0.001	0.598	0.169	0.214	0.018	0.001

Frederickso	0.014	0.679	0.004	0.175	0.08	0.048
WA12930	0.039	0.708	0.063	0.068	0.114	0.009
Germany-11	0.002	0.573	0.094	0.004	0.326	0.001
Imperial	0.002	0.595	0.145	0.228	0.029	0.001
Kombainiesi	0.006	0.42	0.001	0.207	0.365	0.001
B1115	0.16	0.643	0.003	0.002	0.001	0.191
B1067	0.003	0.603	0.054	0.004	0.014	0.321
Yan89110	0.002	0.508	0.01	0.22	0.006	0.255
YYXT	0.033	0.407	0.008	0.18	0.173	0.199
YUQS	0.084	0.49	0.113	0.108	0.001	0.203
Xiaojiang	0.001	0.568	0.002	0.001	0.001	0.427
Gebeina	0.038	0.217	0.009	0.109	0.277	0.35
WA12938	0.019	0.185	0.026	0.108	0.32	0.342
YSM3	0.012	0.423	0.002	0.193	0.003	0.369
Hu93-043	0.018	0.615	0.001	0.033	0.077	0.256
Barlis	0.304	0.002	0.003	0.501	0.003	0.187
HOR3877	0.3	0.011	0.015	0.458	0.005	0.211
keel	0.123	0.002	0.002	0.539	0.333	0.001
Flagship	0.014	0.003	0.006	0.682	0.293	0.003
Barque73	0.008	0.001	0.001	0.705	0.273	0.013
Fleet	0.229	0.001	0.001	0.391	0.375	0.003
WA12920	0.002	0.126	0.029	0.039	0.447	0.357
WA12926	0.002	0.136	0.136	0.145	0.58	0.001
WA12944	0.001	0.001	0.001	0.994	0.001	0.002
WA12946	0.002	0.001	0.002	0.934	0.001	0.061
WA12942	0.003	0.002	0.002	0.829	0.001	0.164
WA12945	0.156	0.002	0.004	0.822	0.006	0.01
WA12948	0.017	0.002	0.001	0.899	0.001	0.08
Mundah	0.035	0.003	0.005	0.578	0.376	0.004
Hindmarsh	0.079	0.002	0.005	0.589	0.324	0.001
HOR8851	0.001	0.005	0.002	0.989	0.003	0
Spanishland	0.001	0.014	0.014	0.967	0.003	0.001
Harington	0.001	0.003	0.001	0.976	0.019	0
Hamelin	0.001	0.002	0.002	0.93	0.065	0.001
Keka	0.074	0.005	0.005	0.892	0.025	0.001
Brindabella	0.326	0.001	0.071	0.443	0.155	0.004
Oram385-2-2	0.31	0.001	0.009	0.349	0.328	0.002
Skiff	0.178	0.004	0.008	0.069	0.65	0.092
Clipper	0.118	0.118	0.004	0.133	0.625	0.002
Sahara	0.003	0.003	0.001	0.502	0.49	0.001
Schooner	0.083	0.036	0.087	0.251	0.534	0.008
Oram257-1	0.18	0.145	0.043	0.009	0.622	0.001
Oram257-3	0.307	0.152	0.005	0.008	0.528	0.001
Antarctia04	0.002	0.088	0.194	0.259	0.454	0.002

FM404	0.002	0.188	0.169	0.017	0.621	0.003
BR1	0.002	0.238	0.196	0.26	0.301	0.003
WA10113	0.127	0.002	0.103	0.122	0.645	0.001
FischersWir	0.006	0.238	0.005	0.134	0.616	0.001
Gairdner	0.005	0.006	0.001	0.301	0.686	0.001
Macquarie	0.067	0.001	0.016	0.321	0.593	0.001
Baudin	0.001	0.001	0.002	0.176	0.818	0.001
116-9707-B	0.001	0.001	0.001	0.002	0.994	0.001
Cheri	0.001	0.005	0.001	0.01	0.982	0.001
Yan90260	0.001	0.001	0.001	0.002	0.994	0.001
115-9505-B	0.001	0.001	0.001	0.001	0.927	0.069
Aurora	0.002	0.006	0.008	0.017	0.893	0.073
Buloke	0.168	0.002	0.009	0.008	0.766	0.048
Lang/Carmen	0.003	0.005	0.011	0.018	0.888	0.075
BoaFe	0.001	0.238	0.001	0.033	0.726	0.001
Cevadade2or	0.001	0.004	0	0.003	0.991	0.001
HOR1448	0.002	0.005	0.001	0.004	0.988	0.001
HOR12779	0.004	0.021	0.001	0.004	0.969	0.002
HOR12522	0.002	0.005	0.001	0.006	0.986	0.001
Carmen	0.001	0.001	0.001	0.001	0.996	0.001
BR2	0.001	0.001	0.001	0.001	0.997	0
Carmen-B	0.002	0.001	0.001	0.001	0.995	0.001
HOR12820	0.002	0.001	0.001	0.001	0.995	0.001
Portuguesel	0.001	0.001	0.001	0.001	0.996	0
Antarctia01	0.001	0.001	0.001	0.001	0.996	0
Horni-Pesek	0.003	0.007	0.009	0.008	0.973	0.001
CI-4196	0.01	0.001	0.003	0.091	0.891	0.004
Ida	0.001	0.012	0.264	0.008	0.714	0.001
WA12949	0.271	0.004	0.143	0.403	0.038	0.14
ACBurman	0.159	0.242	0.35	0.008	0.002	0.239
Oram258-2	0.057	0.204	0.301	0.426	0.01	0.002
Oram258-3	0.112	0.038	0.431	0.399	0.013	0.007
B1100	0.001	0.335	0.386	0.001	0.001	0.275
WA12931	0.001	0.352	0.384	0.001	0.001	0.261
Etu	0.001	0.002	0.609	0.307	0.081	0.001
Nord	0.002	0.044	0.747	0.186	0.022	0
Atoloa	0.087	0.021	0.582	0.307	0.002	0.001
YRJAR	0.14	0.021	0.736	0.099	0.002	0.002
KAJSA	0.001	0.001	0.996	0.001	0.001	0.001
Karin	0.001	0.001	0.994	0.001	0.003	0.001
RIPA	0.001	0.001	0.998	0	0	0
EddaII	0.001	0.001	0.996	0.001	0.001	0.001
WA08649	0	0	0.998	0	0	0
WA08654	0	0	0.998	0	0	0

WA08644	0	0	0.998	0	0	0
WA08655	0	0	0.998	0	0	0
WA08650	0.002	0.001	0.996	0.001	0	0
WA12901	0.141	0.056	0.455	0.174	0.001	0.172
NoireMaroc	0.059	0.123	0.005	0.015	0.305	0.492
WA12917	0.089	0.15	0.007	0.023	0.665	0.066
Russia24	0.332	0.007	0.002	0.239	0.004	0.416
Russia7	0.334	0.004	0.001	0.239	0.004	0.417
Russia39788	0.33	0.003	0.002	0.246	0.006	0.414
WA098	0.134	0.003	0.076	0.165	0.07	0.551
Russian12	0.14	0.005	0.001	0.109	0.429	0.316
CPI-11284-4	0.213	0.014	0.261	0.144	0.068	0.299
YSM1	0.188	0.003	0.156	0.03	0.006	0.617
WA12922	0.142	0.002	0.185	0.19	0.005	0.475
Russia68	0.201	0.002	0.002	0.002	0.098	0.695
Russian68	0.286	0.001	0.002	0.004	0.103	0.604
B1079	0.004	0.001	0.005	0.007	0.003	0.98
Honen	0.001	0.001	0.001	0	0.001	0.997
WA12913	0.003	0.007	0.001	0.001	0.006	0.983
RGZLL	0.001	0	0	0	0.001	0.998
Russian82	0	0	0.001	0	0	0.998
YPSLDM	0.001	0	0	0	0	0.998
CxHKSL	0.001	0.001	0	0.001	0.001	0.997
DYSYH	0.002	0.121	0.004	0.003	0.004	0.865
97IWFBC12	0.133	0.017	0.029	0.058	0.003	0.761
WA12934	0.134	0.306	0.006	0.002	0.013	0.539
YiwuErleng	0.001	0.507	0.001	0.001	0.001	0.49
WA10119	0.341	0.096	0.204	0.001	0.025	0.333
SYR01	0.273	0.035	0.137	0.04	0.15	0.365
WA12947	0.256	0.009	0.229	0.166	0.004	0.337
Russian74	0.253	0.01	0.002	0.123	0.185	0.427
Russian81	0.229	0.005	0.017	0.264	0.096	0.389
W2	0.542	0.001	0.001	0.317	0.001	0.138
ZUG293	0.366	0.005	0.003	0.208	0.002	0.416
Numar	0.412	0.001	0.001	0.262	0.007	0.316
WA12937	0.382	0.005	0.001	0.351	0.036	0.226
Spanishland	0.234	0.113	0.152	0.15	0.342	0.008
WA12914	0.272	0.018	0.176	0.217	0.31	0.007
Spanishland	0.373	0.011	0.169	0.433	0.013	0.001
Dayton	0.42	0.043	0.339	0.071	0.049	0.077
cevadaPreta	0.551	0.133	0.306	0.002	0.002	0.007
HOR2410	0.55	0.15	0.285	0.003	0.001	0.011
Russia	0.439	0.201	0.322	0.023	0.003	0.012
WA12918	0.568	0.028	0.12	0.123	0.01	0.151

Yerong	0.597	0.013	0.228	0.001	0.001	0.16
YF374	0.307	0.002	0.23	0.002	0.323	0.136
HOR8847	0.995	0.002	0.001	0.001	0.001	0.001
Spanishland	0.997	0.001	0.001	0.001	0	0.001
HOR8849	0.997	0.001	0	0	0.001	0.001
Spanishland	0.997	0.001	0	0.001	0	0.001
HOR8850	0.91	0.001	0.001	0.001	0.001	0.088
Spanishland	0.992	0.003	0.001	0.001	0.002	0.001
HOR8846	0.997	0	0.001	0	0.001	0.001
Spanishland	0.997	0.001	0.001	0	0.001	0.001
HOR8848	0.996	0	0.001	0.001	0.001	0.002
Spanishland	0.998	0	0	0	0.001	0
HOR4052	0.901	0.001	0.002	0.014	0.003	0.078
HOR8852	0.893	0.001	0.002	0.098	0.004	0.003
HOR4050	0.859	0.004	0.13	0.003	0.001	0.001
cevadade6or	0.692	0.093	0.207	0.004	0.001	0.002
HOR1590	0.762	0.017	0.196	0.008	0.007	0.01
HOR13461	0.915	0.002	0.001	0.002	0.059	0.021
HOR12517	0.869	0.002	0.006	0.004	0.012	0.107
HOR13446	0.996	0.001	0.001	0.001	0.001	0.001
MAR-82-E113	0.918	0.021	0.006	0.047	0.006	0.001
HOR7327	0.993	0.002	0.001	0.002	0.001	0.001
HOR8842	0.997	0.001	0.001	0.001	0.001	0
Spanishland	0.997	0.001	0.001	0.001	0.001	0
HOR4023	0.975	0.012	0.002	0.007	0.003	0.002
HOR4055	0.928	0.003	0.064	0.001	0.002	0.002
Rosa	0.988	0.001	0.007	0.001	0.002	0.001
HOR13437	0.926	0.007	0.001	0.003	0.035	0.027
HOR13447	0.982	0.004	0.002	0.007	0.002	0.003
morrocanlan	0.989	0.002	0.001	0.003	0.004	0.001
Yambla2	0.001	0.002	0.001	0.991	0.001	0.002
Hor3870	0.001	0.248	0.001	0.033	0.716	0.001
WA12941	0.194	0.001	0.142	0.025	0.007	0.63
B1118	0.525	0.001	0.001	0.327	0.001	0.145
WA12907	0.095	0.772	0.001	0.009	0.002	0.121

Appendix 6.2 Salt tolerance score of 206 barley accessions were grouped into two genotypes according to their Base calls of the marker bpb-9668, bpb-0003, bpb-4285 and bpb-4135. These four QTL showed additive effect with the average tolerance score (2.083) of varieties combining all four tolerance alleles than that of varieties with all susceptible alleles (5.167)

	bPb-9668	bPb-0003	bPb-4285	bPb-4135	bpb-9668/ 0003/ 4285/4135 (aaaa/bbbb)
Genotype a	2.572	2.452	2.597	2.654	2.083
Genotype b	3.532	3.266	3.65	3.025	5.167
b-a	0.96	0.814	1.053	0.371	3.083

Appendix 6.3 List of genes within 15 cM around bpb-9668 at the end of chromosome on 4H

Gene Name	Chromosome	Phy_cM	Gene description
MLOC_70289.1	4	100.2	Glycosylphosphatidylinositol anchor biosynthesis protein 11
MLOC_70290.3	4	100.2	MLo protein
MLOC_14272.1	4	100.6	DET1- and DDB1-associated protein 1
AK367973	4	100.6	Diacylglycerol kinase-like protein
MLOC_13453.1	4	100.6	Pin2-interacting protein x1, putative
MLOC_4076.1	4	100.6	Protein kinase family protein
MLOC_11400.1	4	100.6	WD-repeat protein, putative
AK357206	4	101.1	S-adenosyl-L-methionine-dependent methyltransferase domain-containing protein
AK373775	4	101.3	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit
MLOC_61137.1	4	101.5	Unknown protein
MLOC_55703.1	4	101.6	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
MLOC_13135.1	4	101.6	Callose synthase
MLOC_55702.1	4	101.6	Cytochrome C-type biogenesis protein
AK250747.1	4	101.6	DNA polymerase delta subunit 2
MLOC_78144.1	4	101.6	DNA polymerase delta subunit 2
AK367323	4	101.6	ESTs AU029294(E30104)
AK370202	4	101.6	ESTs AU078251(R0889)
MLOC_68937.1	4	101.6	Exosome complex exonuclease exoribonuclease
MLOC_62333.1	4	101.6	F-box protein
MLOC_20815.2	4	101.6	Glucan synthase-like 3
MLOC_70918.1	4	101.6	Glutathione-regulated potassium-efflux system protein
MLOC_52515.2	4	101.6	Glyceraldehyde-3-phosphate dehydrogenase B
MLOC_36993.2	4	101.6	Hydroxyproline-rich glycoprotein family protein
MLOC_62331.1	4	101.6	Peroxidase
MLOC_62332.1	4	101.6	Peroxidase 54
AK373630	4	101.6	Ring finger protein
AK363664	4	101.6	unknown protein
AK359923	4	101.8	peptide transporter 3 LENGTH=582
MLOC_10725.1	4	101.8	BZIP family transcription factor, putative, expressed
MLOC_10727.2	4	101.8	SMAD/FHA domain-containing protein
AK364947	4	102.0	Anthranilate phosphoribosyltransferase
MLOC_71024.2	4	102.0	CBS domain-containing protein, putative, expressed
MLOC_21112.1	4	102.0	Cell growth defect factor 2
MLOC_10498.1	4	102.0	glucan synthase-like 12 LENGTH=1955
AK373161	4	102.0	Glyoxylate/hydroxypyruvate reductase, putative
MLOC_67679.3	4	102.0	Unknown protein
MLOC_70598.1	4	102.0	Zinc finger protein 3
MLOC_17825.1	4	102.4	DNAJ heat shock N-terminal domain-containing protein LENGTH=726

AK368132	4	103.3	Protein kinase
MLOC_60797.1	4	103.9	Protein of unknown function, DUF538 LENGTH=179
MLOC_37919.1	4	103.9	unknown protein; BEST Arabidopsis thaliana protein match is: unknown protein
MLOC_60135.1	4	103.9	BZIP transcription factor
AK369342	4	103.9	GCN5-related N-acetyltransferase, putative, expressed
AK356026	4	103.9	GDSL esterase/lipase
MLOC_72579.1	4	103.9	Heavy metal transport/detoxification superfamily protein LENGTH=358
MLOC_11974.1	4	103.9	Methyltransferase
MLOC_13076.1	4	103.9	Receptor-like protein kinase
MLOC_57111.1	4	103.9	Unknown protein
AK250812.1	4	103.9	Vacuolar sorting receptor 1, putative
MLOC_57363.1	4	103.9	Vicilin
MLOC_57109.1	4	103.9	Zinc finger protein
MLOC_62434.1	4	104.0	24-sterol C-methyltransferase
AK252978.1	4	104.0	50S ribosomal protein L14
AK359456	4	104.0	Mechanosensitive ion channel
AK362554	4	104.0	Phosphate transporter
AK251272.1	4	104.0	Pre-mRNA-processing factor-like protein
MLOC_13124.4	4	104.0	Protein of unknown function (DUF3527) LENGTH=603
MLOC_75880.1	4	104.0	RNA recognition motif family protein, expressed
AK252954.1	4	104.0	Serine carboxypeptidase, putative
AK370716	4	104.0	Splicing factor u2af large subunit, putative
MLOC_35766.1	4	104.0	Thyroid adenoma associated
MLOC_4875.1	4	104.0	Unknown protein
MLOC_13125.1	4	104.0	Unknown protein
MLOC_22475.2	4	104.5	C2H2-like zinc finger protein LENGTH=173
MLOC_59596.1	4	104.6	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein LENGTH=341
MLOC_57414.1	4	104.6	50S ribosomal protein L11
AK367800	4	104.6	BEL1-like homeodomain protein
AK364918	4	104.6	Beta-amylase
MLOC_3846.1	4	104.6	C2H2 and C2HC zinc fingers superfamily protein LENGTH=191
AK373760	4	104.6	Cysteine-rich repeat secretory protein
MLOC_69029.1	4	104.6	Heterogeneous nuclear ribonucleoprotein D0, putative
AK369536	4	104.6	Homeobox protein knotted-1, putative
AK367522	4	104.6	Inter-alpha-trypsin inhibitor heavy chain H3
AK370626	4	104.6	Inter-alpha-trypsin inhibitor heavy chain H3
AK368348	4	104.6	MADS-box transcription factor
MLOC_66787.1	4	104.6	PLAC8-like protein
AK366779	4	104.6	Protein kinase, putative, expressed
AK364785	4	104.6	SNARE associated Golgi protein family LENGTH=320
MLOC_18334.2	4	104.6	Sugar transporter, putative
AK370403	4	104.6	WD-repeat cell cycle regulatory protein

MLOC_11235.1	4	104.6	Zinc finger protein, putative
MLOC_74586.1	4	104.8	Plant protein of unknown function (DUF946) LENGTH=567
MLOC_75889.3	4	105.0	Pectin lyase-like superfamily protein LENGTH=476
MLOC_55155.1	4	105.5	Unknown protein
AK367470	4	106.0	Heavy metal transport/detoxification superfamily protein LENGTH=183
AK365681	4	107.4	Hydrolase
MLOC_61612.1	4	107.6	12-oxophytodienoate reductase 2
MLOC_38948.1	4	108.8	Kinase-like protein
AK249039.1	4	109.2	Peroxidase 66
MLOC_70775.3	4	109.2	Unknown protein
MLOC_8706.1	4	109.2	DNA helicase, putative
MLOC_70109.1	4	109.2	Double Clp-N motif-containing P-loop nucleoside triphosphate hydrolases superfamily protein
MLOC_65132.1	4	109.2	Formyltetrahydrofolate deformylase
MLOC_16631.1	4	109.2	Plant protein of unknown function (DUF828) with plant pleckstrin homology-like region
MLOC_65128.1	4	109.2	Plant protein of unknown function (DUF828) with plant pleckstrin homology-like region
MLOC_81568.1	4	109.2	Ulp1 protease family, C-terminal catalytic domain, putative
AK376104	4	110.2	Alpha-1,2-fucosidase
MLOC_71237.1	4	110.2	Aquaporin 1
AK248994.1	4	110.2	Cell cycle control protein
MLOC_65155.1	4	110.2	Leucine-rich receptor-like protein kinase family protein LENGTH=1173
MLOC_13188.2	4	110.2	Membrane protein insertase YidC 2
MLOC_65156.2	4	110.2	Pentatricopeptide repeat-containing protein, putative
MLOC_53267.2	4	110.2	Periplasmic binding protein
MLOC_18098.1	4	110.2	Receptor-kinase, putative
MLOC_18292.1	4	110.2	Remorin family protein LENGTH=486
MLOC_60639.1	4	110.4	Ataxin-10
MLOC_50290.1	4	110.4	Cyclin dependent kinase A
MLOC_37087.1	4	110.4	glucan synthase-like 10 LENGTH=1890
MLOC_53568.1	4	110.4	Hydroxymethylglutaryl-CoA synthase, putative, expressed
AK355828	4	110.4	O-methyltransferase
MLOC_60640.1	4	110.4	Oxidoreductase, 2OG-Fe(II) oxygenase family family
MLOC_50291.1	4	110.4	Post-GPI attachment to proteins factor
MLOC_68648.2	4	111.2	Unknown protein
AK358799	4	111.3	26S proteasome non-ATPase regulatory subunit 11
AK360929	4	111.3	Aspartate--tRNA ligase-like protein
MLOC_64528.1	4	111.3	Chaperone protein clpB
MLOC_73329.1	4	111.3	Charged multivesicular body protein 3
AK365216	4	111.3	Disease resistance-responsive (dirigent-like protein) family protein LENGTH=193
MLOC_64534.1	4	111.3	DNA repair protein
MLOC_59840.1	4	111.3	Ectonucleoside triphosphate diphosphohydrolase 5
MLOC_80133.4	4	111.3	FAR1-related sequence 6 LENGTH=703

MLOC_59888.1	4	111.3	F-box family protein, putative, expressed
MLOC_55415.5	4	111.3	Fructosamine kinase family protein
AK367749	4	111.3	Germin-like protein 4d
MLOC_55416.1	4	111.3	Pentatricopeptide repeat-containing protein
MLOC_64533.1	4	111.3	peptidyl-prolyl cis-trans isomerases LENGTH=343
AK363167	4	111.3	Peroxisomal membrane protein 11-1
MLOC_60423.2	4	111.3	Plant protein of unknown function (DUF863) LENGTH=483
MLOC_36901.3	4	111.3	Protein phosphatase 2c, putative
MLOC_36900.2	4	111.3	RING finger and WD repeat domain-containing protein
MLOC_55097.3	4	111.3	RING finger and WD repeat domain-containing protein
AK356496	4	111.3	Xyloglucan endotransglucosylase/hydrolase protein 5
MLOC_66801.1	4	111.9	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
MLOC_58176.2	4	111.9	Protein kinase superfamily protein LENGTH=824
AK252710.1	4	112.1	Cell number regulator 6
MLOC_53551.1	4	112.1	Chromodomain-helicase-DNA-binding protein 1-like
MLOC_29110.1	4	112.1	Coatomer alpha subunit
MLOC_66457.1	4	112.1	General transcription factor IIH subunit
MLOC_17751.1	4	112.1	Leucine-rich repeat receptor-like protein kinase
AK249011.1	4	112.1	Oxidoreductase, zinc-binding dehydrogenase family protein, expressed
MLOC_72732.3	4	112.1	Palmitoyl protein thioesterase containing protein, expressed
MLOC_66975.3	4	112.1	Sugar transporter, putative
MLOC_49761.1	4	112.2	Uncharacterised protein family (UPF0497) LENGTH=152
MLOC_64782.2	4	112.3	Beta-fructofuranosidase
MLOC_7084.3	4	112.3	Dual protein phosphatase 4
MLOC_70063.2	4	112.3	GHMP kinase protein
AK354381	4	112.3	Hydrolase, alpha/beta fold family protein, expressed
MLOC_37252.1	4	112.3	Pentatricopeptide repeat-containing protein, putative
AK365896	4	112.3	Unknown protein
AK361453	4	112.3	WD-repeat protein, putative
AK374366	4	112.3	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AK360661	4	112.5	ATP-dependent Clp protease proteolytic subunit
AK251931.1	4	112.5	Branched-chain-amino-acid aminotransferase
MLOC_58890.1	4	112.5	BTB/POZ domain containing protein
MLOC_70762.1	4	112.5	FAD-binding Berberine family protein LENGTH=535
MLOC_18417.1	4	112.5	Fatty acyl coA reductase
MLOC_58892.3	4	112.5	Myosin heavy chain-related protein LENGTH=853
MLOC_58889.1	4	112.5	NBS-LRR disease resistance protein
MLOC_5021.1	4	112.5	Respiratory burst oxidase-like protein B2
AK373965	4	113.0	Phosphatidylinositol N-acetyglucosaminlytransferase subunit P-like protein
MLOC_15230.1	4	113.1	2-dehydro-3-deoxyphosphogluconate aldolase/4-hydroxy-2-oxoglutarate aldolase
MLOC_10539.3	4	113.1	ABC(ATP-binding) family transporter
AK375220	4	113.1	Expansin B4

MLOC_45717.1	4	113.1	Pollen allergen-like protein
MLOC_79748.3	4	113.1	Retinol dehydrogenase 12
AK365879	4	113.3	Xyloglucan endotransglucosylase/hydrolase protein 32
MLOC_33809.1	4	113.7	60 kDa jasmonate-induced protein
AK372562	4	113.7	60 kDa jasmonate-induced protein, putative
AK251203.1	4	113.7	Acid phosphatase
AK364105	4	113.7	Armadillo/beta-catenin-like repeat family protein
MLOC_10843.1	4	113.7	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
MLOC_16300.1	4	113.7	Ectonucleoside triphosphate diphosphohydrolase 5
AK376221	4	113.7	Expansin protein
MLOC_22160.1	4	113.7	HXXXD-type acyl-transferase family protein LENGTH=428
MLOC_71487.4	4	113.7	PAIR1 protein, putative, expressed
AK369262	4	113.7	Phosphatidylinositol-4-phosphate 5-kinase family protein, putative, expressed
AK368847	4	113.7	Protein of unknown function (DUF3527) LENGTH=694
MLOC_60426.3	4	113.7	RNA polymerase II transcription mediators LENGTH=2253
MLOC_24239.1	4	113.7	Transferase family protein, expressed
MLOC_10437.1	4	113.7	tRNA/rRNA methyltransferase (SpoU) family protein
MLOC_51915.2	4	113.7	Ubiquitin-protein ligase, putative
MLOC_48416.1	4	113.7	Unknown protein
MLOC_60425.1	4	113.7	UPF0187-containing protein
MLOC_7409.1	4	113.8	Small nuclear ribonucleoprotein-like protein
AK365195	4	114.9	basic helix-loop-helix (bHLH) DNA-binding superfamily protein LENGTH=264
AK248746.1	4	115.2	basic helix-loop-helix (bHLH) DNA-binding superfamily protein LENGTH=181
AK251005.1	4	115.2	Tubulin beta chain, putative